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DEVELOPMENT OF AUTOCHTHONOUS PROBIOTIC CANDIDATES FOR TILAPIA AQUACULTURE

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UNIVERSITY OF
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A thesis submitted to the University of Plymouth
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ABSTRACT

This programme of work sought to develop autochthonous probiotic solutions for tilapia aquaculture. Initial work began with the isolation of isolate 34 bacterial cultures from the tilapia intestine, which were tested for probiotic potential *in vitro*. Fifteen isolates displayed positive probiotic properties in *in vitro* assays. The selection of high potential probiotic candidates was based on multi-parameter properties using the Z−score method, which ranked isolates identified as *Bacillus* sp. CHP02 (Z score = 1.48), *Bacillus* sp. RP01 (1.14) and *Bacillus* sp. RP00 (1.09) as having the greatest potential. These isolates, along with *Enterobacter* sp. NP02 (0.50), were then assessed for their efficacy as probiotic candidates *in vivo*. Six experimental groups: T1: (*Bacillus* sp. CHP02 + a commercial feed), T2 (*Bacillus* sp. RP01 + a commercial feed), T3 (*Bacillus* sp. RP00 + a commercial feed), T4 (*Enterobacter* sp. NP02 + a commercial feed), T5 (*P. acidilactici* + a commercial feed) and T6 (only + a commercial feed) were designed for evaluation in both fry and on-growing stages of tilapia. *Bacillus* sp. RP01 application to feeds induced positive effects on tilapia larvae including improved body weight, total weight gain, average daily growth, specific growth rate and resistance to *A. hydrophila* challenge. However, these beneficial effects were not observed when applied in on-growing sized tilapia. The results suggest that the Z-score method could be used to select high potential of autochthonous probiotics for fry, but the applicability in the current research programme was less robust at later life stages. It is hypothesised that different probiotic strains may be required for application during different life stages, which may reflect the different physiologies of tilapia, and their likely differing microbiomes, at different life histories. Further research is required to select probiotics by using re-isolation and both *in vitro* and *in vivo* trials across the whole tilapia production cycle.

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List of abbreviations

A.	*Aeromonas*
Acid.	*Acidovorax*
Acin.	*Acinetobacter*
ADG	Average daily growth
Agro.	*Agrobacterium*
AIT	Asian Institute of Technology
Ano.	*Anoxybacillus*
B.	*Bacillus*
Bre.	*Brevundimonas*
Bur.	*Burkhoderia*
C.	*Cronobacter*
Car.	*Carnobacterium*
Ce.	*Cetobacterium*
cfu	Colony forming unit
Chro.	*Chromobacterium*
Chry.	*Chryseobacterium*
Ci.	*Citrobacter*
Clos.	*Clostridium*
Cor.	*Corynebacterium*
Cur.	*Curtobacterium*
dpf	Day post fertilization
dph	Day post-hatch
E.	*Escherichia*

Ed.	*Edwardsiella*
En.	*Enterococcus*
Ent.	*Enterobacter*
Entero.	*Enterobacteriaceae*
FCR	Feed conversion ratio
Fla.	*Flavimonas*
Flav.	*Flavobacterium*
GIT	Gastrointestinal tract
IM	Intra-muscular
IP	Intra-peritoneal
IW	Increasing weight
K	Fulton’s condition factor
KMITL	King Mongkut's Institute of Technology Ladkrabang
L.	*Lactococcus*
Lac.	*Lactobacillus*
Leuc.	*Leuconostocmesenteroides*
Lis.	*Listeria*
mt	Million tonnes
Mac.	*Macrococcus*
Mi.	*Micrococcus*
My.	*Mycobacterium*
Pas.	*Pasteurella*
Pho.	*Photobacterium*

Ple.	*Plesiomonas*
Pro.	*Providential*
Pseu.	*Pseudomonas*
R.	*Roseobacter*
Rho.	*Rhodopseudomonas*
RIL	Relative intestinal length
S.	*Streptococcus*
Sac.	*Saccharomyces*
Sal.	*Salmonella*
Ser.	*Serratia*
SGR	Specific growth rate
She.	*Shewanella*
SPG	Specific growth rate
SR	Survival rate
Stap.	*Staphylococcus*
TCC	Total colony counts
TL	Total length
TLG	Total length gain
V.	*Vibrio*
W	Weight
WG	Weight gain
Yer.	*Yersinia*

Dedication

To

Mum and Dad, please relax in peace.

In my heart, everything is done in the right way as you did.

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I would like to dedicate this thesis to my lovely children, who are Naruwan (Ping-Ping), Kawan (Sun-Sun) and Kawin (Tian-Tain) Panakulchaiwit. You make me to have a high power to do everything.

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Author's declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee.

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Publications:

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Rodiles, A., Rawling, M.D., Peggs, D.L., Pereira, G.V., Voller, S., Yomla, R., Standen, B.T., Bowyer, P. and Merrifield D.L. (2018). Probiotic Applications for Finfish Aquaculture. In: Di Gioia D., Biavati B. (eds) Probiotics and Prebiotics in Animal Health and Food Safety. Springer, Cham, 197-217. DOI: https://doi.org/10.1007/978-3-319-71950-4_8

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Yomla, R., Merrifield, D. and Davie, S. (2015). Investigating the safety of potential probiotic candidates isolated from the GI tract of tilapia in vitro, on, oral presentation in The 2nd International Symposium on Agricultural Technology Global agriculture Trends for Sustainability, July 1-3, 2015 Pattaya, Thailand

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Chapter 1

General introduction

Aquaculture provides a significant and important source of protein for supporting the human population. Total production was 66.6 million tonnes (mt) in 2012, which constituted 24.7 mt of marine aquaculture and 41.9 mt of inland aquaculture (approximately 4 mt of tilapia production). Forecasts suggest that aquaculture productions in 2030 may increase to 101.2 mt with tilapia accounting for about 30% of volume. The world population in 2012 was 7.06 billion, and may increase to 8 billion in 2030 (FAO, 2014; www.prb.org, 2016). Therefore, aquaculture production is very important to provide food for people worldwide. Tilapia species are considered to be 'the fish for next-generation aquaculture' (Yue *et al*., 2016), which are cultured worldwide.

1.1 Tilapia aquaculture

Tilapia aquaculture is distributed worldwide in more than 130 countries, including China, Indonesia, Philippines, Thailand, Vietnam, Egypt, Columbia, Bangladesh, Brazil, and Egypt (FAO, 2014). During the first quarter of 2015, Europe imported a total of 7,702 tonnes of frozen tilapia, which were produced in China, Vietnam, Thailand, and Myanmar (www.fao.org, 2016).

Tilapia were originally introduced to Thailand when fifty Nile tilapia (*Oreochromis niloticus*) as a royal tribute from the Emperor of Japan were sent to H.M. King of Thailand on March 25, 1965 (Department of Fisheries, 2011). These fish were bred at the Chitralada garden in the Dusit Palace. Then, fish larvae were transferred to the Department of Fisheries at the Bangkhen University for research on feeding and breeding techniques and larvae were then distributed to agricultural

farmers. Tilapias have many beneficial properties such as good qualities and taste, easy to rear, rapid growth, and a high fecundity (Bhujel, 2013). Since 2003, the department of fisheries (DOF) has planned a project on "good aquaculture practice" to promote tilapia aquaculture after as economic species to lead tilapia productions having good qualities and safe for consumers (Lawonyawut, 2007).

The tilapia production cycle may be separated into two parts: 1) larval phase and 2) on-growing phase (Figure 1.1). The larval phase includes broodstock management, hatching process, nursing systems and male production. A main problem in the farms during crop production is facing with different sizes of larval growth associating with early maturing of tilapia. Therefore sex reversal using synthetic androgenic hormone (17 methyl-testosterone) is used to treat in the fifth stage of larval tilapia (Figure 1.2) changing phenotypes to male characterization, which improves the consistency of tilapia production (www.fao.org, 2016). The on-growing phase usually rears both in the earthen pond and cages within or without the closed system.

Generally, pathogenic *Aeromonas* spp. are distributed in aquaculture systems and freshwater fish; these may often be present in the gastro-intestinal tract (GIT) of healthy fish (Nedoluha and Westhoff, 1997; Spanggaard *et al*., 2000; Molinari *et al*., 2003; Al-Harbi and Uddin, 2004, 2005a&b; Blancheton *et al*., 2012). Causing pathogenic loads of 10^5 cfu.g^{-1} in an aquaculture system can induce fish diseases (Buller, 2004), which might be the effect of the dysbiosis of beneficial and pathogenic microbes (Ringø *et al*., 2007). Farmers use a combination of antimicrobials, parasiticides, chemicals, drugs, feed additives, and probiotics, to prevent or treat disease outbreaks, and to promote healthy fish (FAO, 2014; Rico *et al*., 2013). Farmers are increasingly under pressure today to improve ecological sustainability by reducing the use of drugs and chemicals (Volpe *et al*., 2010; Levin and Stevenson, 2012; HLPE, 2014). Probiotics have therefore been suggested to be an environmentally friendly solution for aquaculture (Denev, 2008).

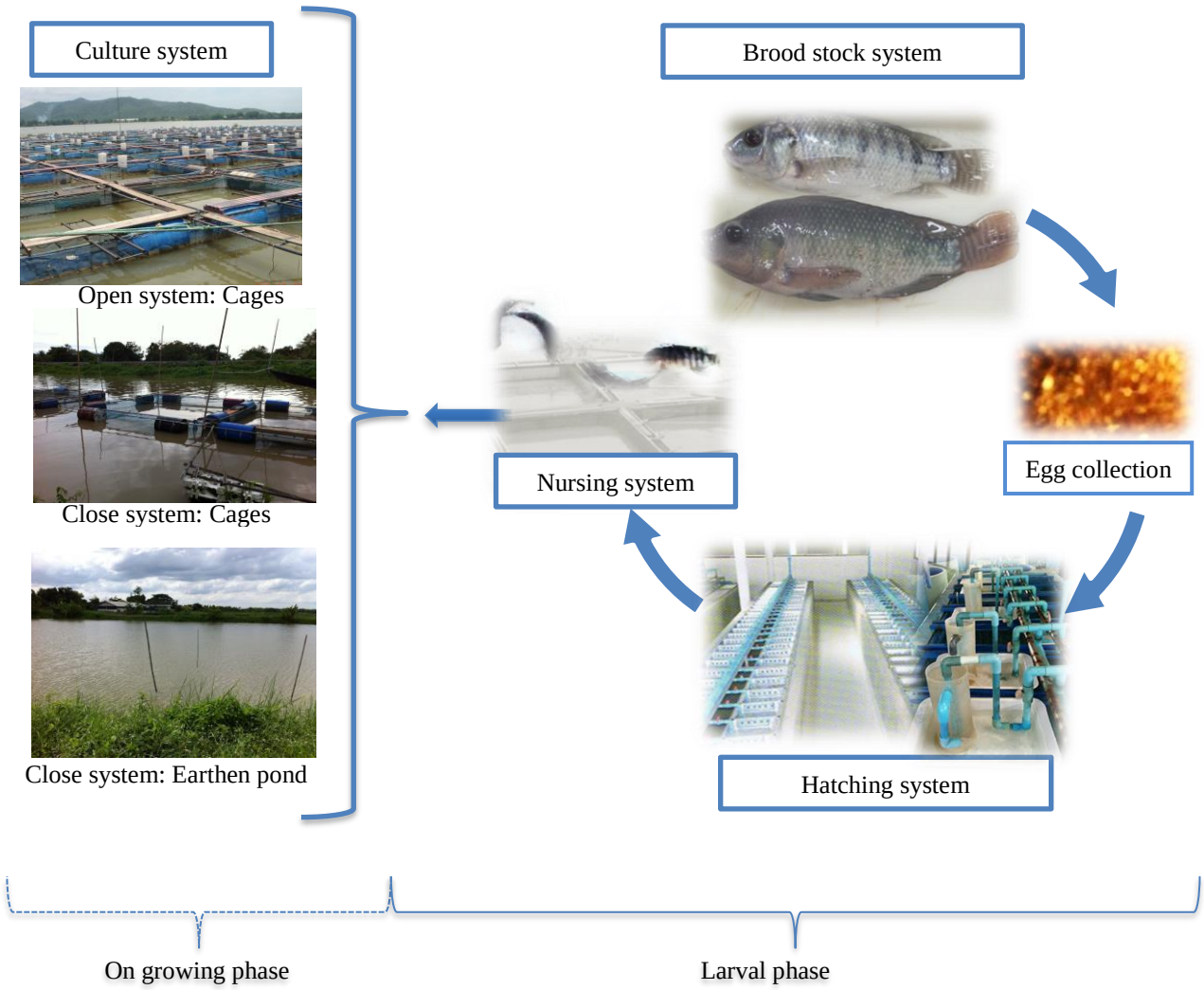


Figure 1.1 Overview of tilapia production methods in Thailand.

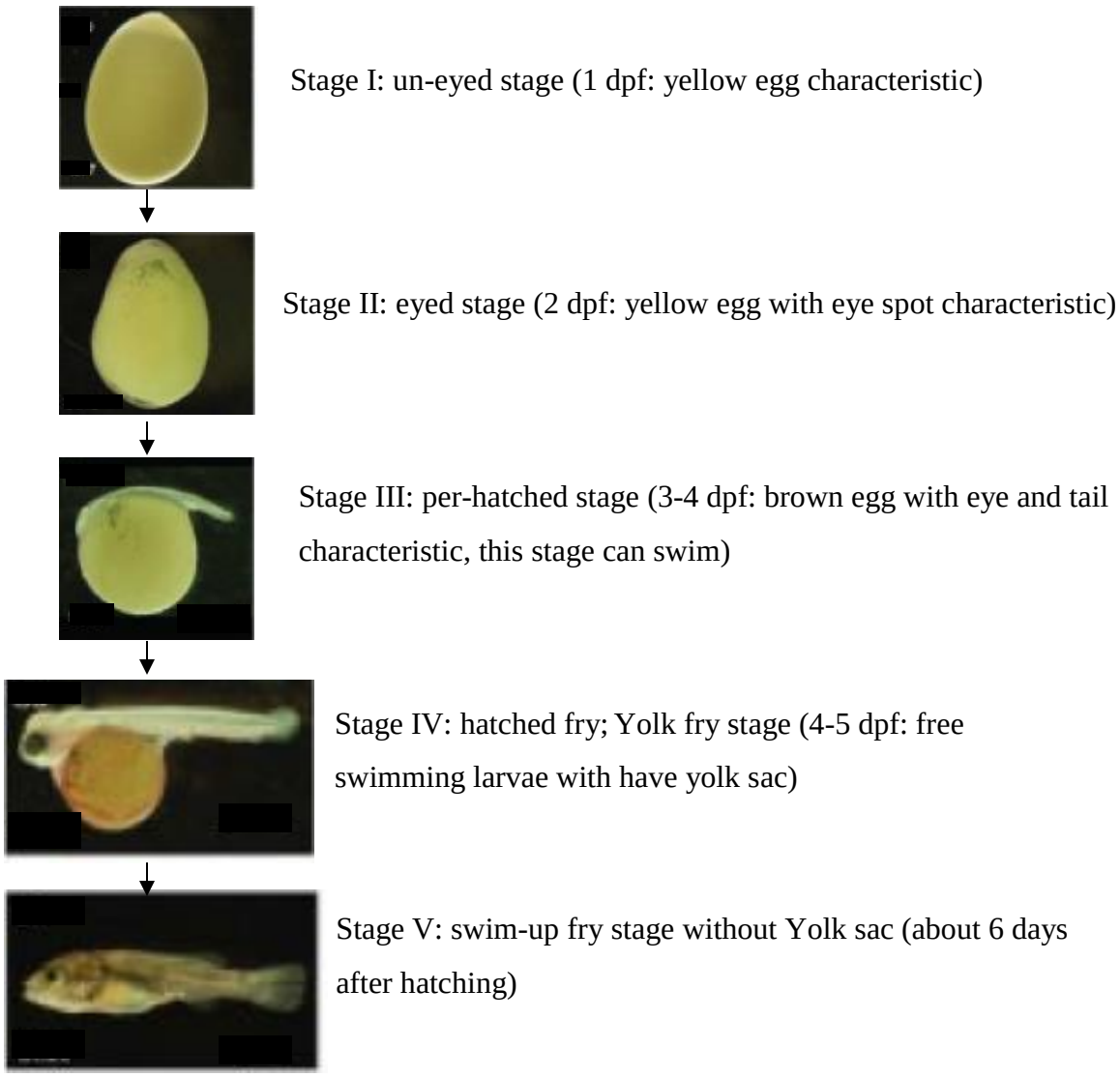


Figure 1.2 Nile tilapia larval development.

Source: Modified from Fujimura and Okada (2007)

1.2 Probiotics for aquaculture

1.2.1 Definitions

Probiotics are defined as live microbes introduced into the gastrointestinal tract by administration via the food or water system, which promote internal microbial balance to promote good health (Parker, 1974; Fuller, 1989; Fuller, 1992; Gatesoupe, 1999; Verschuere *et al*., 2000). The definition

given by the FDA (2006) was ‘live microorganisms that are ingested with the intention of providing a health benefit’, while the FAO/WTO (2006) defined it as ‘live microorganisms when consumed in adequate amounts as part of food confer a health benefit on the host’.

In 2008, probiotics were suggested for use in aquaculture as an environmentally friendly method in disease prevention (Wang *et al*., 2008). Another definition is ‘microorganisms administered orally leading to health benefits, are used extensively in aquaculture for disease control, notably against bacterial diseases’ (Newaj-Fyzul *et al*., 2014). Furthermore, I suggest the meaning of probiotic microbes that are beneficial for the host and the user (b), environmentally friendly (e), sustainable aquaculture (s) and trust of stakeholders (t).

Nowadays, commercial probiotics are popular selling in powder form such as Alibio®, Bactocell PA10 MD, Bactocell® PA 10, Biomate SF-20, Biogen®, BioPlus® 2B, Cernivet®, Levucell SB 20, Sigma, Sporolac, and Toyocerin® (Chang *et al*., 2002; Raida *et al*., 2002; Shelby *et al*., 2006; EL-Haroun *et al*., 2006; Aly *et al*., 2008b; Castex *et al*., 2010; Harikrishnan *et al*., 2010; Luis-Villaseñor *et al*., 2013). These probiotics are familiar in many aquatic farms such as tilapia, shrimp, and pangasius farms in Asia (Figure 1.3).

Several reviews reported that probiotic usages in aquaculture supported various benefits, which included improvements of growth performances, disease resistances, immune enhancement, health status, balancing function mechanisms of fishes, sustainability of gut microbes, water quality (as bioremediation to improve water quality and break down nutrient), and to enrich the nutrients in zooplankton (Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000; Marques *et al*., 2005; Kesarcodi-Watson *et al*., 2008; Wang *et al*., 2008a; Merrifield *et al*., 2010; Haché and Plante, 2011). The usage of probiotics can impact both gut microbes and water microbes, which have supported fish health.

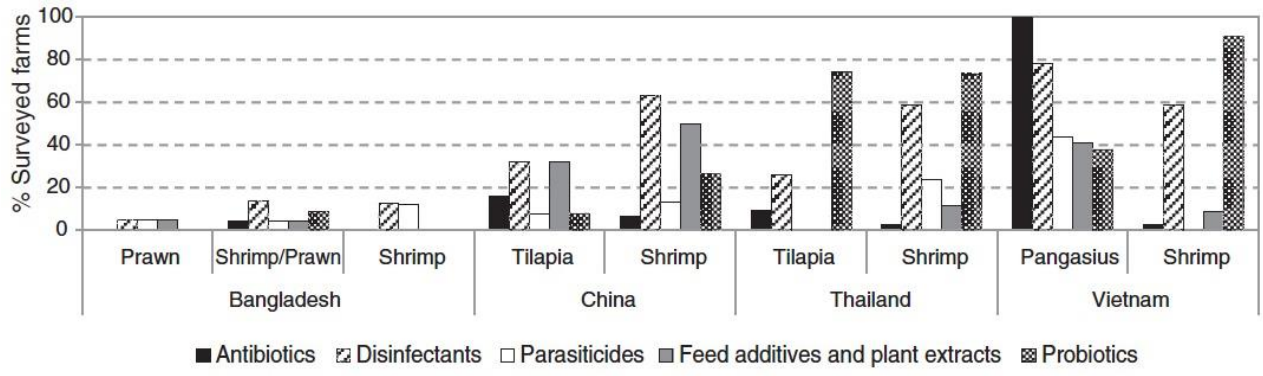


Figure 1.3 Percentage of farmers that use antibiotics, disinfectants, parasiticides, feed additives and plant extracts, and probiotics in each of the studied farm groups in Asia.

Source: Rico *et al.* (2013)

1.2.2 Sources of bacterial probiotics

Generally, microbes are occurring in human, aquatic animals, snow, soils, sediments, groundwater, freshwater and seawater and different numbers of bacteria (10^2 to 10^{11} cfu.g^{-1}) are observed in biotic and abiotic environments (Torsvik *et al*., 1990; Al-Harbi and Uddin, 2003; Segee, 2005; Senders *et al*., 2007; Liu *et al*., 2010; Nimrat *et al*., 2012; Tiago and VerÍssimo, 2012). Exogenous bacteria (from air, soil, human etc.) may enter water systems. These microbes could change populations as 'microbial communities developing in the culture water' (Verschuere *et al*., 2000), which can lead different bacteria to colonize in the GIT of aquatic animals. The typical levels of cultivable bacteria reported in different sections of fish trials are displayed in Table 1.1.

The intestinal tract of aquatic animals typical contains around 10^2 to 10^9 cfu.g^{-1} of microbial loads (Spanggaard *et al*., 2000; Al-Harbi and Uddin, 2003, 2004 & 2005a; Molinari *et al*., 2003; Brunt and Austin, 2005; Pond *et al*., 2006; Balcázar *et al*., 2007; Wu *et al*., 2010). Bacterial loads (cfu.g^{-1}) in tilapia system have been estimated to vary from 10^4 to 10^9 in the GIT, 10^5 to 10^8 on the gills, 10^3 to 10^7 in water culture, and 10^6 to 10^8 in pond sediment, while pathogenic loads in the GIT of tilapia and water culture were found to be 10^1 to 10^3 (Figure 1.4).

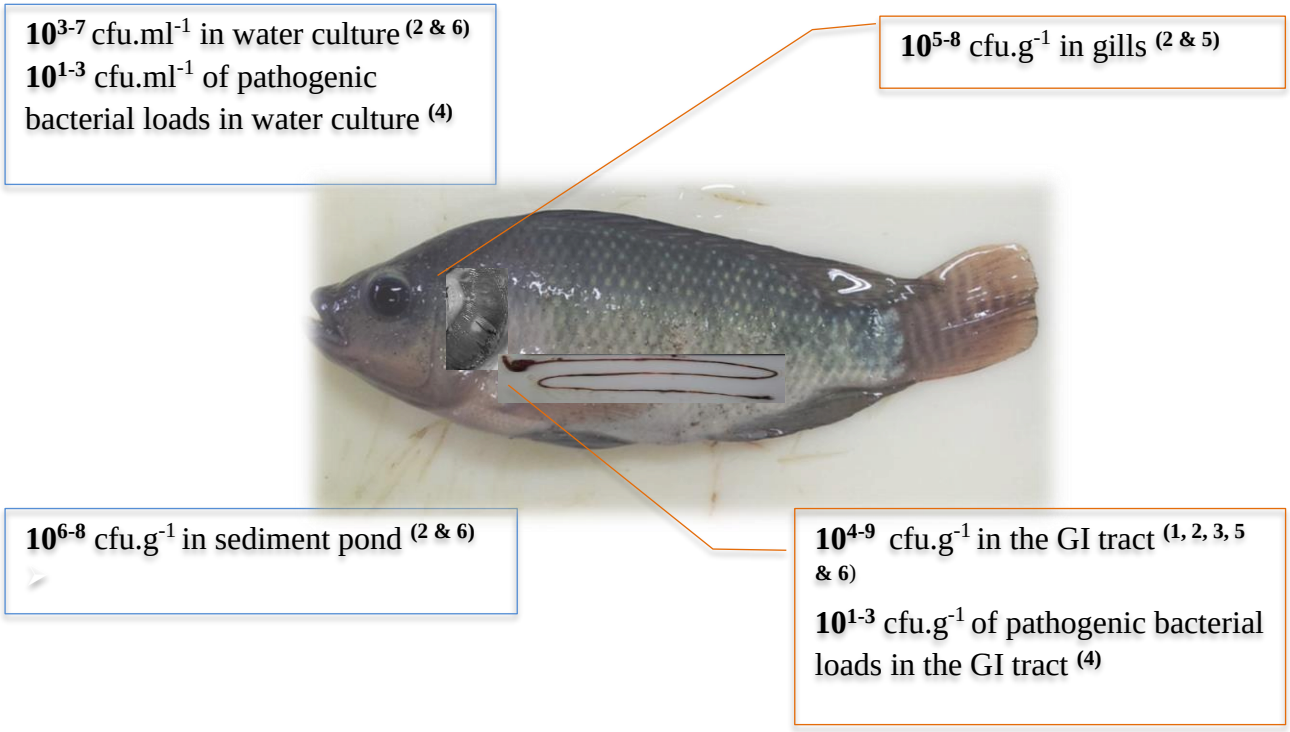


Figure 1.4 Reported cultivable bacterial levels associated with tilapia.

Sources: [1] Molinari *et al*., (2003); [2] Al-Harbi and Uddin (2003): [3] Al-Harbi and Uddin (2004); [4] Boari *et al*. (2008); [5] Shinkafi and Ukwaja (2010); [6] Del'Duca *et al*. (2015)

Table 1.1 Examples of bacterial identifications from different aquaculture components.

Bacterial identification	Study technique	Sources	References
Bre. vesicularis, *Methylobacterium* spp., *Mi. luteus* and *Pseu. pickettii*	Systematic bacteriological study, API 20NE, and BIOLOG system	Aquatic biofilm	Buswell *et al*. (1997)
Aeromonas sp., *Acinetobacter* sp., *Carnobacterium* sp., *Citrobactor* sp., *Plesiomonas* sp., *Pseudomonas* sp., *Proteus* sp., *Shewanella* sp., and *Serratia* sp.	Systematic bacteriological study, RAPD analysis and 16S rRNA sequencing	The intestinal tract of rainbow trout (*Oncorhynchus mykiss*)	Spanggaard *et al*. (2000)
Ae. hydrophila, *A. veronii*, *Bur. cepacia*, *Chro. violaceum*, *Ci. freundii*, *E. coli*, *Fla. oryzihabitans*, and *Ple. shigelloides*	Systematic bacteriological study and focused on Enterobacteriaceae and gram-negative	The gastrointestinal tract of Nile tilapia	Molinari *et al*. (2003)
A. hydrophila, *Bacillus* sp., *Cellulomonas* sp., *Cor. afermentas*, *Cor. urealyticum*, *Cur. pusillum*, *E. coli*, *Flavobacterium* sp., *Micrococcus* sp., *Pasteurella* sp., *P. pnemotropica*, *Pho. damselae*, *Psudomonas* sp., *P. fluorescens*, *Salmonella* sp., *Ser. liquefaciens*, *She. putrefaciens*, *Staphylococcus* sp., *Streptococcus* sp. and *V. cholera*	Systematic bacteriological study, API 20E, API 20STREP, API 50CD and BIOLOG system	The intestinal tract of hybrid tilapia	Al-Harbi and Uddin (2004)
A. hydrophila, *Cor. afermentas*, *Cor. urealyticum*, *E. coli*, *Flavobacterium* sp., *Micrococus* sp., *Pasteurella* sp., *Photo. damsella*, *Pseudomonas* sp., *Ser. liquifaciens*, *She. putrefaciens*, *Staphylococcus* sp., *Streptococcus* sp., and *V. cholerae*	Systematic bacteriological study, API 20E, and BIOLOG system	The intestinal tract of hybrid tilapia	Al-Harbi and Uddin (2003)
A. hydrophila, *Cor. urealyticum*, *Cor. liquifaciens*, *E. coli*, *Flavobacterium* sp., *Pasteurella* sp., *Photo. damsella*, *Pseudomonas* sp., and *She. putrefaciens*,	Systematic bacteriological study, API 20E, and BIOLOG system	The gills of hybrid tilapia	Al-Harbi and Uddin (2003)
A. hydrophila, *Acin. delafieldii*, *Cor. urealyticum*, *E. coli*, *Flavobacterium* sp., *Micrococus* sp., *Pasteurella* sp., *Photo. damsella*, *Pseudomonas* sp., *Ser. liquifaciens*, *She. putrefaciens*, *Staphylococcus* sp., *Streptococcus* sp., and *V. cholerae*	Systematic bacteriological study, API 20E, and BIOLOG system	The earthen pond water of hybrid tilapia rearing	Al-Harbi and Uddin (2003)
Alcaligenes sp., *Pseudomonas* spp., *Pseudoalteromonas* sp., *Roseobacter* spp., *R. gallaciensis*, *R. denitrificans*, *R. litoralis*	Systematic bacteriological study, RAPD analysis and 16S rRNA sequencing	Turbot larvae (*Scophthalmus maximus*) rearing units	Hjelm *et al*. (2004)

Table 1.1 *Continued…*

Bacterial identification	Study technique	Sources	References
A. hydrophila, Acid. delafieldii, Bur. glumae, Cor. urealyticum, Cor. liquifaciens, E. coli, Flavobacterium sp., *Micrococus* sp., *Pasteurella* sp., *Pseudomonas* sp., *Pseu. fluorescens, Ser. liquifaciens, She. putrefaciens,* , *Streptococcus* sp., and *V. cholerae*	Systematic bacteriological study, API 20E, and BIOLOG system	The earthen pond sediment of hybrid tilapia rearing	**Al-Harbi and Uddin** (2003)
Aeromonas sp., *A. veronii, A. sobria, Car. piscicola, Clos. gasigenes, En. amnigenus, Plesiomonas* sp., *Ple. shigelloides, She. putrifaciens* and *Plateurella* sp.,	BIOLOG system, API strips, RFLP analysis and 16S rRNA sequencing	The intestinal tract of rainbow trout (*Oncorhynchus mykiss*)	Pond *et al*. (2006)
A. allosaccharophila, A. punctata, A. veronii, Acinetobacter sp., *Agro. tumefaciens, Ano. flavithermus, Ce. somerae, Ce. ceti, Chry. haifense, Clostridium* spp., *Corynebacterium* sp., *Enterobacter* sp., *Ent. ictaluri, En. saccharominimus, E. coli, Ed. ictaluri, Herbaspirillum* sp., *L. garvieae, Ochobactrum* sp., *Microbacterium lacticum, Moraxella* sp., *Myroides odoratimimus, Ple. shigelloides, Ralstonia pickettii, Shewanella* sp., *Sh. putrefaciens, Sphingomonas* sp., *V. cholerae, Yer. ruckeri*	16S rDNA sequencing and Direct DNA extraction from the intestinal samples to clone libraries	The intestinal contents and mucous of yellow catfish (*Pelteobagrus fulvidraco*)	Wu *et al*. (2010)
A. hydrophila, A. allosaccharophilla, Ple. shigelloides, Shewanellaceae sp., Shewanella sp., and *She. purtrefaciens*	PCR-DGGE analysis and 16S rDNA sequencing	The intestinal tract of beluga (*Huso huso*)	Salma *et al*. (2011)
Acientobacter sp., *Ac. junii, Bacillus* sp., *Bre. diminuta, Cetobacterium* spp., *Enterobacteriaceae bacterium, E. coli, Serratia* sp., and *S. proteamaculans*	16S rDNA V3 PCR-DGGE fingerprints	The intestinal tract of hybrid tilapia	He *et al*. (2013)
A. hydrophila, Paracoccus chinensis, and Gramma poteobacterium	16S rDNA V3 PCR-DGGE fingerprints	The intestinal tract of hybrid tilapia	Ren *et al*. (2013)

The potential of probiotic candidates has been assessed from different areas such as semi-intensive systems, floating cages in a river, farm culture, and natural lakes (Molinari *et al.*, 2003; Hagi *et al*., 2004; Hjelm *et al*., 2004; Chantharasophon *et al*., 2011; Chemlal-Kherraz *et al*., 2012; Sugita e*t al*., 2012), where microbes isolated outside the host are termed allochthonous or exogenous and microbes are isolated from inside the host are termed autochthonous or indigenous (Ringø *et al*., 2016).

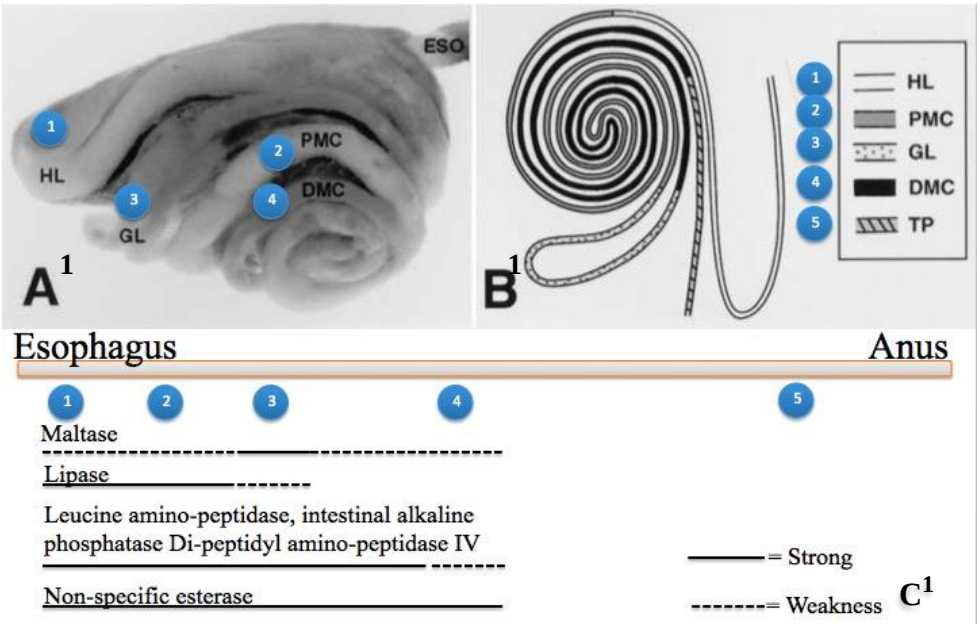
1.2.3 Probiotics can improve gut microecology and improve host growth performance

Many vitamins, fatty acids and amino acids, enzymes, are produced by bacteria such as amylase by *Aeromonas* spp., *B. subtilis*, Bacteridaceae, *Clostridium* spp., *Lactobacillus plantarum* and *Staphylococcus* sp., protease by *B. subtilis* and *Lactobacillus plantarum*, *Staphylococcus* sp. and cellulase by *B. subtilis*, *Lactobacillus plantarum* and *Staphylococcus* sp. (Sugita *et al*., 1997; Balcazar *et al*., 2006; Eissa *et al*., 2010; Efendi and Yusra, 2014; Sarkar and Ghosh, 2014). According to Mondal *et al*., 2008), the tilapia GIT contains amylolytic bacteria (7.3×10^3 cfu.g^{-1}), cellulolytic bacteria (1.5×10^3 cfu.g^{-1}) and proteolytic bacteria (9.0×10^3 cfu.g^{-1}). Similarly, Sarkar and Ghosh (2014) observed different bacterial groups in different positions of the tilapia gut, which are dominantly proteolytic bacteria (7.3×10^3 cfu.g^{-1}), cellulolytic bacteria (5.0×10^3 cfu.g^{-1}) in the hindgut gut and amylolytic bacteria (7.3×10^3 cfu.g^{-1}) at the foregut and the other bacterial groups (2.3 to 2.7×10^3 cfu.g^{-1}) in the mid-gut.

A rule of the enzymatic digestibility in the intestine of tilapia has the effect on feed intakes to break down into molecules. Several enzymes are different releases between the foregut to the mid-gut (Figure 1.6), however, non-enzyme activities display in the hindgut (Figure 1.6A-C & 1.6D1-D3). *Aeromonas* spp. can produce amylase to digest carbohydrates, which is a primary source providing greater energy in omnivorous (Molinari *et al*., 2003). Probiotic supplements in fish feed have been reported to increase bacteria loads in the GIT (Figure 1.6D1-D3; Jatobá *et al*., 2011), which may improve digestibility and improve growth performances. Microbial varieties were reported to find

different bacteria such as *A. hydrophila, Ple. shigelloides, Fla. oryzihabitans, E. coli* and *Chro. violaceum* in stomach, *A. veronii, Ple. shigelloides, Chro. violaceum* and unidentified sp in the mid-gut and *A. veronii, Bur. cepacia, Ci. freundii*, *Ple. shigelloides* and unidentified species in the posterior gut of tilapia of tilapia culturing in the semi-intensive system (Molinari *et al*., 2003).

Gastrointestinal bacterial loading and/or activity may be influenced by diet. Previous studies reported that a single dose of probiotic candidates as *B. amyloliquefaciens, B. firmus, B. pumilus, B. subtilis, Citro. freundii, L. acidophilus, Lactobacillus* sp. and *P. acidilactici* at concentrations of $10^{6 - 12}$ cfu.g^{-1} diet have been supplemented in tilapia feed and the optimal period of probiotic feeding is around 4-8 weeks (Aly *et al*., 2008a,b&c; Nouh *et al*., 2009; He *et al*., 2013; Liu *et al*., 2013; Stenden *et al*., 2013). A commercial probiotic (Biogens: *B. subtilis* Natto; not less than 6×10^7.g^{-1}) *B. amyloliquefaciens* (10^8 cfu. g^{-1} diet) were suggested to mix in fish feed. They provided positive effects on FCR (EL-Haroun *et al*., 2006; Ridha and Azad, 2012). According to He *et al*., (2013) both allochthonous and autochthonous *Bacillus* were only observed in the probiotic group. The gut microbes may directly affect to nutritional digestibility associating growth performances in tilapia.



	Fore gut	Mid-gut	Hind-gut	
Control:	2.1×10^4	2.0×10^5	3.0×10^4	**D1[2]**
Probiotic:	1.6×10^6	1.3×10^7	5.8×10^7	
Control:	3.5×10^4	4.8×10^4	5.2×10^4	**D2[2]**
Probiotic:	1.1×10^6	7.2×10^6	9.3×10^6	
Control:	1.7×10^4	1.1×10^5	2.8×10^4	**D3[2]**
Probiotic:	3.3×10^6	5.5×10^5	1.0×10^6	
Natural resource	3.7×10^4	8.0×10^4	1.3×10^5	**E[3]**

Figure 1.5 Enzymatic activities and different number of the gut microbiota in Nile tilapia; **A[1]**: right view, **B[1]**: drawing ventral view (1. HL: hepatic loop, 2. PMC: proximal major coil, 3. GL: gastric loop, 4. DMC: distal major coil and 5. TP: terminal portion of the intestine), **C[1]**: enzyme activities, **D1-D3**: bacterial loads ($cfl.ml^{-1}.cm^{-1}$) in the tilapia, **D1[2]**: 99 days fed probiotic, **D2[2]**: 40 days fed without probiotic, **D3[2]**: 61 days fed without probiotic, **E[3]**: tilapia from natural resource.

Sources: [1] Tengjaroenkul *et al*. (2000); [2] Ridha and Azad (2012); [3] Sarkar and Ghosh, 2014

1.3 How to prove the efficiency of novel probiotic for aquaculture use

The "Guideline for the Evaluation of Probiotics in Food" is a global standard, which suggests how to evaluate probiotics both *in vitro* and *in vivo* before using in human (FAO/WTO, 2006). Consequently, potential probiotics use in aquaculture may also follow this guideline with some parameters adjusted to fit with aquatic animals. Basically, probiotics are declared as safe to use, which have information backgrounds of genotype, phenotype, and characterization for users. In *in vitro* trials, several properties of probiotics are usually evaluated acidic and bile salt tolerances, adherences and antimicrobial activities and then potential of probiotic candidates are based on the results *in vitro* trials, finally these probiotics are tested in living aquatic animal. Probiotics for aquatic animals should be tested as described in the following sections.

1.3.1 *In vitro* trials

In vitro trial can lead to reduce the cost testing and sample sizes of living animals for *in vivo* studies. Often, pathogen antagonism tests are considered a suitable initial screening method to test antagonistic activities (Aly *et al*., 2008b; Balcázar *et al*., 2008; El-Rhman *et al*., 2009; Chemlal-Kherraz *et al*., 2012). Parameters such as blood hemolysis, antibiotic resistance, adherence assays, pH and bile salt tolerances, and the other properties are used investigation for screening the potential of probiotics *in vitro* trials.

1.3.1.1 Pathogenic inhibitions

Probiotics are presumed to produce compounds such as bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxides, which can inhibit pathogens (Ringø and Gatesoupe, 1998; Gomez-Gil *et al*., 2000; Verschuere *et al*., 2000; Lara-Flores *et al*., 2003; Shelby *et al*., 2006; Abdel-Tawwab *et al*., 2008; El-Rhman *et al*., 2009; Nayak, 2010; Ringø *et al*., 2010; Ridha and Azad; 2012). Bacterial pathogens are illustrated in Table 1.1, which are used to indicate potential of allochthonous/ autochthonous probiotic candidates for using in tilapia.

Probiotics can produce substances or compounds that inhibit pathogenic bacterial growth. Therefore, many studies use agar plates to evaluate their potential. A simple technique is “spot on the lawn”. This technique begins with a pathogenic bacterium swabbing on TSA plate. The plate is incubated and then the potential probiotic candidate is used to spot on this agar plate (Vine *et al*, 2004; Chantharasophon *et al*., 2011). A double-layer method is a quick method to screen bacterial isolates, which uses a single colony of isolates to culture on TSA plate. Then, growing colonies are removed and added semi-solid TSA containing the bacterial pathogen to cover this plate (Del'Duca *et al*., 2013). A well diffusion is used fresh bacterial cells or bacterial supernatant into holes on the plate, which spread with a pathogen (Hjelm *et al*., 2004; Hai *et al*., 2007; Apún-Molina *et al*., 2009; Chemlal-Kherraz *et al*., 2012; Hamdan *et al*., 2016).

A familiar protocol is agar diffusion, which begins to use potential probiotic spreading overnight on TSA agar and pathogenic testing is used to spot culture on this plate (Aly *et al*., 2008a; Aly *et al*., 2008c; Eissa *et al*., 2014). Another technique is a disc diffusion method, which uses a paper disc to immerse in cell–free supernatant of cultural bacterial broth of isolates. A dried agar plate with a pathogen is prepared and then these paper discs are put on this plate (Hai *et al*., 2007; Balcázar *et al*., 2008). The quorum quenching is used to demonstrate the potential of probiotics to inhibit violacein, which produced by *C. ciolaceum* (Villamil *et al*., 2014).

Finally, a ‘cross streaking method’ is used isolated bacteria to streak in the center of the agar plate and then removed and killed bacterial growth following to use pathogenic bacteria culture on this plate (Hai *et al*., 2007). The appearance of clear zone of these methods is used to indicate the potential of isolates inhibited pathogens.

Table 1.2 Exemplary pathogens use to test with potential isolates *in vitro* trials.

Pathogens and bacterial testing	Potential probiotics	Sources	Antibacterial activities	References
Gram-negative and rod shape: *A. hydrophila*	*B. firmus, B. pumilus and Citrobactor freundii*	The internal organs of Nile tilapia	Isolates can inhibit pathogen	Aly *et al*., 2008a
Gram-negative and rod shape: *A. hydrophila*	*Micro. luteus* and *Pseudomonas* sp.	The gonads and intestine of Nile tilapia	Isolates can inhibit pathogen	El-Rhman *et al*., 2009
Gram-negative and rod shape: *A. hydrophila*	*Bacilllus* UBRU4	The intestinal tract of Nile tilapia	Inhibit to pathogen	Chantharasophon *et al*., 2011
Gram-negative and rod shape: *A. hydrophila, E. coli, Ed. tarda, Fla. branchiophilum, Pseu. aeruginosa, Pseu. fluorescens, Salmonella.* sp. and *Shigella* sp.; Gram-positive and cocci shape: *Streptococcus* sp.	*B. subtilis*	The GIT of three species of Indian major carps.	Inhibit to all pathogens	Nayak and Mukherjee, 2011
Gram-negative and rod shape: *E. coli, Pseudomonas* sp.; Gram-positive and cocci shape: *Stap. aureus Streptococcus* sp. and	Two strains of *Lactobacillus* spp.	The intestinal tract of Nile tilapia	LAB strain BLT31 only displays non-inhibition to *E. coli*	Chemlal-Kherraz *et al*., 2012
Gram-negative and curved-rod shape: *Vibrio* sp.	*Pediococcus pentosaceus* (LAB 37 and LAB 1-6) and *Pediococcus* sp. (LAB 35),	The intestinal tract of tilapia	Isolates display non-inhibition to pathogen	Cota-Gastélum *et al*, 2013
Gram-negative and rod shape: *A. hydrophila, Ed. tarda, Pseu. fluorescens and Pseu. putida*; Gram-positive and cocci shape: *Ent. faecalis*	*Bacillus* sp. (1: autochthonous probiotic) and *Enterococcus* sp. (2: allochthonous probiotic)	The intestine of tilapia (1) and the pond's sediment (2)	*Bacillus* sp. and *Enterococcus* sp. can inhibit all pathogens acceptable *Ent. faecalis*	Del'Duca *et al*., 2013
Gram-negative and rod shape: *Ed. tarda*	*L. lactis* subsp. *Lactis*	The intestinal tract of freshwater fish	Inhibit to pathogen	Loh *et al*., 2014
Gram-negative and rod shape: *E. coli* and *Klebsiella* sp. Gram-positive and cocci shape: *Staphylococcus* sp.; Gram-positive and rod shape: *Bacillus*.	Two LAB strains	The GIT of tilapia and channa	Inhibit to all pathogens	Vijayaram and Kannan, 2014

1.3.1.2 Blood hemolysis

Bacterial pathogens such as *Aeromonas* spp. and *Streptococcus* spp. are normally found in the GIT of fish (Marcel *et al*., 2013). They contain virulence genes (haemolysin and aerolysin) to hemolyse blood cells (Yogananth *et al*., 2009; Marcel *et al*., 2013). Hemolytic activities be after can assessed using several blood types such as human blood, horse blood, sheep blood, blood fishes, and shrimp hemolymph (Apún-Molina *et al*., 2009; Leyva-Madrigal *et al*., 2011; Leyva-Madrigal *et al*., 2011; Nayak and Mukherjee, 2011; Cota-Gastélum *et al*, 2013; Muñoz-Atienza *et al.*, 2013; Loh *et al*., 2014; Vijayaram and Kannan, 2014; Hamdam et al., 2016). Bacterial isolates as *Bacillus* spp., *Ci. freundii*, *Lac. plantarum,* and *Lac. casei* have been proved non-harmful on blood hemolysis (Aly *et al*., 2008a; Apún-Molina *et al*., 2009; Chantharasophon *et al*., 2011; Chemlal-Kherraz *et al*., 2012).

1.3.1.3 Antibiotic resistances

Microorganisms can produce antibiotics, which are natural substances to prevent or inhibit pathogenic microbes (EC 1831/2003, 2003; Serrano, 2005; Rico *et al*., 2013). Both natural and synthesised antibiotics have been used so much in aquaculture. Consequently, the prevalence of antimicrobial residues has been remaining in aquatic animals and natural water environments (Petersen and Dalsgaard, 2003; Michel *et al*., 2003; Kemper, 2008; Baquero *et al*, 2008; Singh *et al*., 2009; Krishnika and Ramasamy, 2013, Nhung *et al*., 2015). Microbes can display both specific resistance and multi-resistance. These resistance genes are inherited from generation to generation and might transfer to other bacterial species or strains through horizontal gene transfer. For instance, microbial pathogens such as *E. coli*, *Enterococcus* spp., and *Salmonella* spp. have been detected resistant genes (Petersen and Dalsgaard, 2003; Michel *et al*., 2007).

Several articles reported that probiotic strains as *Bacillus* spp. show resistance to penicillin and kanamycin, some LAB strains displayed on multiple resistances as cefoxitin, chloramphenicol, penicillin, kanamycin, and oxacillin (Mourad and Nour-Eddine, 2006; Chantharasophon *et al.*,

2011; Chemlal-Kherraz *et al*., 2012). It has therefore been suggested that probiotics should be free of plasmid encoded antibiotic resistance genes" and add a citation for this.

1.3.1.4 Adhesion/aggregation/colonization

Bacterial colonization is considered a prerequisite of potential probiotics (Ringø and Gatesoupe, 1998). Several adhesion assays are used to explore high potential probiotics to adhere to fish mucous, epithelial cells, semi-solid media, hard substrate, gelatin, polystyrene and bovine serum albumin (Pan *et al*., 2008; Geraylou *et al*., 2014; Preito *et al*., 2014). Furthermore, adhesion has been evaluated in terms of bacterial adherence to solvents, hydrophobicity, or biofilm formation (Abdulla *et al*., 2014; Preito *et al*., 2014).

An auto-aggregation assay is used to evaluate bacteria adhesion between cells to cells within strains or species (Pen *et al*., 2008; Lazado *et al*., 2011; Abdulla *et al*., 2014), while adhesion of cells to cells of different strains (between isolates and pathogen) is called a co-aggregation (Grześkowiak *et al*., 2012; Abdulla *et al*., 2014). A co-aggregation method or co-culture method may be used to assess competitive adhesion between bacterial isolate and pathogen (Pan *et al*., 2008; Lazado *et al*., 2011). These assays might be examined in buffer solvents or broth media.

Several articles reported that the ability of bacterial adhesions is determined with different substrates such as the intestinal epithelial cells (IEC), fish mucous, and the epithelial cell line (Pan *et al*., 2008; Grześkowiak *et al*., 2011; Lazado *et al*., 2011; Geraylou *et al*., 2014; Preito *et al*., 2014; Etyemez and Balcazar, 2016). The host mucous has been used to demonstrate the adhesive efficiency of probiotic candidates (Grześkowiak *et al*., 2011). In some studies of these articles, bacterial isolates have demonstrated displaying high growth rate on mucous than the other medium culture. At the same of Geraylou *et al*. (2014) reported that different isolates were displayed differences of the adhesive properties both media culture and on mucous. Adhesive potentials can also be determined as microbial adhesion to solvents (MATS) or bacterial adhesion to hydrocarbons (BATH) or hydrophobicity (Rosenberg and Rosenberg, 1985; Collado *et al*. 2008). The solvents

used include chloroform, ethyl acetate, n-hexadecane, n-octane, octonol, p-xylene, polystyrene and xylene (Van der Mei *et al*., 1995; Kos *et al*., 2003; Balcázar *et al*., 2007; Wang *et al*., 2007; Pan *et al*., 2008; Grześkowiak *et al*., 2012; Geraylou *et al*., 2014; Preito *et al*., 2014). Furthermore, BATH technique as cell surface hydrophobicity is used non-polar solvents for estimating the adhesive potential of probiotic candidates (Bellon-Fontaine *et al*., 1996).

The estimation of bacterial changes may be achieved by many techniques such as conventional methods such as the plate count technique (Pan *et al*., 2008; Preito *et al*., 2014; Widanarni *et al*., 2015; Etyemez and Balcazar, 2016), and a direct bacterial count (Lazado *et al*., 2011), an optical density (a micro-plate reader) or bacterial-labeled radioactivity and auto-fluorescence monitoring (Balcázar *et al*., 2007; Grześkowiak *et al*., 2011; Geraylou *et al*., 2014; Pham *et al*., 2014).

1.3.1.5 Tolerance of gastrointestinal tract conditions

The GIT of fish is a relatively harsh environment comprised of digestive enzymes, pH variations and bile salts. The mucous cells in the GIT of Nile tilapia have been observed to resist acidity associating with pH ranging from 1.58 to 5.0 in the stomach (Morrison and Wright, 1999; Hlophe *et al.*, 2013). Moreover, pH changes ranging 1 to 7.8 in the intestinal tract of fish are occurring during the pepsin activity and pH higher than 7.8 during lipid activity (Bone and Moore, 2008; Hagey *et al*., 2010). The potential of isolates to tolerate with low pH is important for selecting probiotics. The pH of 2 has been found the effect on the survival rate of probiotics, whilst bile salts were found a few effects on probiotic mortality (Mourad and Nour-Eddine, 2006; Balcázar *et al*., 2008; Nayak and Mukherjee, 2011; Chemlal-Kherraz *et al*., 2012; Geraylou *et al*., 2014).

1.3.2 The selection of potential probiotic using *in vitro* trials

Using several numbers of probiotics testing *in vivo* trial may be related to use facilities, materials, high number of lab animals and high budget. Referring to the 3Rs having three components of reducing, refinement, and replacing animals are suggested for researcher in response these components as ethical awareness (Festing and Altman, 2002). Then, *in vitro* trials are very important as a pre-study experiment without using lab animals.

Various articles have distributed different methods to select probiotics. For instance, pathogenic activities are the initial examination and then followed with safety testing (Aly *et al*., 2008; Balcázar *et al*., 2008; El-Rhman *et al*., 2009), blood hemolysis and pathogenic inhibition (Aly *et al*., 2008; El-Rhman *et al*., 2009; Chantharasophon *et al*., 2011; *Gobinath et al*., 2012; Del'Duca *et al*., 2013), only the property of bacterial aggregation (Grześkowiak, *et al*., 2012) or used pathogenic inhibition and adhesive potentials (Etyemez and Balcazar, 2016). The correlation between cell surface hydrophobicity and auto-aggregation has been pointed to select the potential of probiotics (Wang *et al*., 2007). The simplest method to select high potentials of probiotics may use a few parameters and use a few isolates in the initial study. The selection of probiotics might be using different parameters for evaluating probiotic potentials, which are listed in Table 1.2.

Multi-parameters such as pathogenic antagonism, susceptibility to antibiotics, ability to produce lactic acid and pH, and bile salt tolerances have been used to select probiotics (Chemlal-Kherraz *et al*., 2012). Muñoz-Atienza *et al*., (2013) reported that the selection of probiotics based on the results of hemolysin production, antibiotic susceptibility, bile salt deconjugation, mucin degradation, enzymatic activities, and antibiotic resistance gene. Some evidences have found different findings of each isolate displaying different parameters such as cell surface properties (Collado *et al*., 2008), auto-aggregation and co-aggregation (Collado *et al*., 2008, Grześkowiak *et al*., 2011&2012), and adhesive capacities to different substrates (Balcázar *et al*., 2007; Vendrell *et al*., 2009; Grześkowiak *et al*., 2011). Moreover, Vine *et al*., (2004) suggested the ranking index (RI), which used parameters

of doubling time and lag period of bacterial growth *in vitro* testing. This model has the assumption that bacterium having a short lag period and short doubling time were displayed a high opportunity of probiotic properties (low RI). Different bacterial strains may display varieties of findings and each strain might possibly occur different results from different parameters. Then, the point is how to use all parameters to calculate together with systematic analysis for selecting high potentials of probiotics.

Table 1.3 Summary of probiotic selection for tilapia using different *in vitro* criteria.

Potential probiotic	Sources	Criteria for evaluation of potential probiotic *in vitro* trials																References
		1. Evaluation of pathogenic inhibition on agar plate studies						2. Assessment of safety to use			3. Evaluations for supporting adhesion							
		1.1	1.2	1.3	1.4	1.5	1A	2.1	2.2	2A	3.1	3.2	3.3	3.4	4.1	4.2	4.3	
Bacilllus UBRU4	The GIT of Nile tilapia	−	+	−	−	−	1	+	+	3	−	−	−	−	−	−	+	Chantharasophon *et al*., 2011
B. firmus, *B. pumilus* and *Citrobactor freundii*	The internal organs of Nile tilapia	+	−	−	−	−	1	−	−	−	−	−	−	−	−	−	−	Aly *et al*., 2008a
Micrococcus luteus and *Pseudomonas*	The organ of Nile tilapia	+	−	−	−	−	1	−	−	−	−	−	−	−	−	−	−	El-Rhman *et al*., 2009
Enterococcus sp.(allochthonous probiotic) and *Bacillus* sp. (autochthonnous probiotic)	Exogenous and endogenous bacteria of tilapia	−	−	−	+	−	5	−	−	−	−	−	−	−	−	−	−	Del'Duca *et al*., 2013
Bacilli *sp.* and LAB strain	Exogenous/ endogenous bacteria tilapia	+	−	−	−	−	1	+	−	−	−	−	−	−	−	−	+	Apún-Molina *et al*., 2009
B. mojavensis B191	The intestinal mucous of Nile tilapia	+	−	−	−	+	2	−	−	−	+	−	−	−	−	−	−	Etyemez and Balcazar, 2016
L. lactis subsp. *Lactis* CF4MRS	The GIT of freshwater fish	−	+	−	−	−	−	+	+	6	−	−	−	+	−	−	−	Loh *et al*., 2014
Lact. plantarum AH78	Marine bacteria	+	−	−	−	−	6	+	+	10	−	−	−	−	−	−	−	Hamdan *et al*., 2016
Lactobacillus spp. BLT1 and BLT3	The GIT of Nile tilapia	+	−	−	−	−	4	−	+	11	−	−	−	−	+	+	+	Chemlal-Kherraz *et al*., 2012
Pediococcus sp. and *P. pentosaceus*	The GIT of tilapia	+	−	−	−	−	1	+	−	−	−	+	−	−	−	−	+	Cota-Gastélum *et al*, 2013
Two *Lactobacilli* strains	The GIT of tilapia and channa	−	−	+	−	−	4	+	+	3	−	−	+	−	−	−	−	Vijayaram and Kannan, 2014
B. subtilis	The GIT of three species of Indian major carps	−	−	+	+	−	10	+	+	10	−	−	−	−	−	−	−	Nayak and Mukherjee, 2011

1.1 Agar diffusion/ agar well diffusion/ well diffusion/ spent culture liquid; 1.2 Cross streak; 1.3 Disc diffusion; 1.4 Double layer; 1.5 Spot on lawn; 1A Totaled pathogenic strains

2.1 Blood hemolytic testing; 2.2 Antibiotic resistances; 2A Totaled antibiotic disc

3.1 Adhesion to many substrates (cells, mucous, semi-solid media, hard substrate, solvents)/biofilm formation; 3.2 Bacterial adherence to hydrocarbons; 3.3 Auto-aggregation; 3.4 Co-aggregation/ co-culture (solvent, broth);
4.1 pH tolerance; 4.2 Bile salts tolerance; 4.3 Others: cultural conditions, growth kinetics/ growth, enzymatic production

1.3.3 *In vivo* trials

The evaluation of potential probiotics for tilapia in *in vivo* trials was carried out for 2 to 34 weeks. The initial weight of tilapia was varied from 1.0 g to 185.0 g. The gap of stocking densities was found to be less than 1.0 $g.l^{-1}$ to a high density (50 $g.l^{-1}$). The difference of both feeding frequency and feeding ration has been found and protein contents in basal diets display varying from 25 to 55 percentages. These data are represented in Table 1.3.

The feed ratio and frequency for larval tilapia weighting 1−2 g should be around 10−15% body weight per day and 3−8 tpd depending on cultural rearing (www.fao.org, 2016). Fish feeds could be reduced and adjusted to upon tilapia growing. In addition, protein levels in tilapia feed and stocking density might considerable awareness during culture conditions (Abdel-Tawwab, 2012). Typically, probiotic concentrations of 10^{6-7} $cfu.g^{-1}$ use to mix with fish feed, however, the variation of bacterial cells might be ranging from 10^5 $cfu.g^{-1}$ to 10^{14} (Table 1.3).

The safety to use of potential probiotics before testing *in vivo* trials have been reported that probiotic cells are injected into the fish IP to the observed mortality without severe symptoms of pathogens (Aly *et al*., 2008a,b; El-Rhman *et al*., 2009; Eissa *et al*., 2010). Several parameters such as growth performances, disease resistances, and different parameters in the GIT as hematological studies, histological analysis, and microbial changes have been used to evaluate potential probiotic for tilapia following:

1.3.3.1 Growth performances

Growth performances may be categorized into main parameters and minor parameters. The main parameters consisting of feed conversion ratio, specific growth rate and daily weight growth are routine measurements for monitoring by farmers. Other parameters (Table 1.3) such as hematological studies, histological studies and molecular studies may provide as minor growth performances, which processed in laboratory facilities, which required many chemicals, instruments, and materials.

Several articles have reported that potential probiotics display the positive effect on growth performances (Aly *et al*., 2008c; Eissa and Abou-ElGheit, 2014). For instance, the autochthonous probiotic of the LAB strain use to mix in feed, the other autochthonous Bacilli strain is added to the rearing system and both probiotics were combined together for testing in tilapia culture. These are found high performances of the final weight, absolute growth, absolute growth rate and specific growth than the control group (Apún-Molina *et al*., 2009). Many minor parameters of probiotic testing have shown a higher performance in probiotic groups than the control group. In addition, probiotic potential has been reported to provide high efficiency of low protein diets, which may reduce the production cost (Ghazalah *et al*., 2010). Moreover, different probiotic properties (a high adhesion and low adhesion) have shown different effects on FCR and weight gain of hybrid tilapia (Liu *et al*., 2013).

Conversely, the efficiency of probiotics has been demonstrated without high performances at extreme conditions of stock densities and protein levels (Lara-Flores *et al*., 2003). The negative effect of probiotics has been reporting lower growth in the tilapia fry stage (Shelby *et al.,* 2006, He *et al*., 2013; Standen *et al*., 2013).

1.3.3.2 Pathogenic resistances

Pathogenic resistances are usually tested with fish finishing probiotic-feeding. These findings might be found the positive or negative effects of potential probiotics. Probiotics have been reported to provide a higher survival rate than the control group in several articles (Lara-Flores *et al*., 2003; Aly *et al*., 2008c). Examples, fish fed probiotics at a concentration of $10^{5\text{-}9}$ cells for fifteen days displaying against pathogens (Nouh *et al*., 2009; Liu *et al*., 2013: Villamil *et al*., 2014). Conversely, fish fed probiotics at a concentration of $10^{5\text{-}9}$ cells for eight weeks without providing the survival rate than the control groups (Shelby *et al.,* 2006; Apún-Molina *et al*., 2009; El-Rhman *et al*., 2009; He *et al*., 2013).

1.3.3.3 Bacterial changes in the fish intestine

Both quantitative and qualitative methods are studied to monitor bacterial changes in the GIT of tilapia during the probiotic-feeding and finishing feeding probiotics. Differences of quantitative methods consisting of direct count in media cultures, direct cell count and bacterial labeled-fluorescent probe to detect the specific DNA sequence on chromosomes of the GIT for estimating bacterial abundances (Del'Duca *et al*., 2013). Qualitative methods, such as genomic studies as 16S rDNA V3 region (Ferguson *et al*., 2010), are useful for bacterial probiotic monitoring, polymerase chain reaction - denaturing gradient gel electrophoresis (He *et al*., 2013; Liu *et al*., 2013; Standen *et al*., 2015), which allowed bacterial DNA fragments of the gut microbes to be separated on the basis of sequence differences containing guanines (G) and cytosines (C) by using in polyacrylamide gels containing of denaturing agents, and high-throughput sequencing (Adeoye *et al*., 2016) is meta-genomic microbes in the gut. These are used to identify bacterial species in the tilapia GIT.

Certainly, fish feed probiotics are occurring massive probiotics than the control group (Ferguson *et al*., 2010; Standen *et al*., 2015&2016), whilst microbial loads in the fish GI might be found not different between probiotic and without probiotics, which displayed approximately 10^{6-7} cfu.g^{-1} (Ferguson *et al*., 2010; Liu *et al*., 2013; Standen *et al*., 2013). Using high adhesive probiotic (Lactobacilli) at a concentration more than 10^{7} cfu.g^{-1} diet to feed in fish for 10 days has been provided these bacteria to adhere at the GIT of tilapia (Liu *et al*., 2013). *Bacillus* spp. was displayed in the GIT after fish fed probiotic for 8 weeks, which used an agar plate technique (Standen *et al*., 2015). Probiotics were demonstrated to persist in the GIT along fewer three weeks after finishing probiotic feed (Ferguson *et al*., 2010; Standen *et al*., 2015). Therefore, probiotic cells in fish feed may adhere in the GIT, which could be monitored by using different techniques.

The most important article reported by Del'Duca *et al*., (2013), who used fluorescent-labeled bacterial count in probiotic groups (*Bacillus* sp., *Enterococcus* sp. and combined two potential probiotics) and the control group. Pathogenic bacteria consisting of *Aeromonas* sp. ($0.35\pm0.17\times10^{6}$

cells.g^{-1}) and *Pseudomonas fluorescens* (0.51±0.27×10^6 cells.g^{-1}) displayed higher in the control group and also found in three probiotic groups. The *Enterococcus* group provided high abundances (0.42±0.15×10^6 cells.g^{-1}) in both a single dose and mixing with *Bacillus* sp., however, this bacterium also occurred in all group studies. Similarly, *Bacillus* sp. displayed the highest abundance (1.0±0.47×10^6 cells.g^{-1}) in the *Bacillus* group and high abundance (0.63±0.18×10^6 cells.g^{-1}) in the combined probiotics. Furthermore *Bacillus* sp. seemed to be high (≈0.45±0.13×10^6 cells. g^{-1}) in both the *Enterococcus* and without probiotics.

The potential of probiotics accompanying with the GI microbes has been examined, which found dominant bacteria of *Acinetobacter* spp., *Enterobacteriaceae bacterium*, *Serratia* sp. *Cetobacterium* sp. in probiotic groups and without probiotic (He *et al*., 2013). Some articles have used a high-throughput sequencing analysis to identify bacteria of digesta samples, which identified as *Burkholderia*, *Leuconostoc*, *Acinetobacter*, *Legionella*, *Lactobacillus*, *Corynebacterium*, *Firmicutes*, *Proteobacteria*, and *Cyanobacteria*, which were dominant in the control group. In addition, some bacteria as *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Nitrospirae*, and *Spirochaetes* were found in both treatments (Standen *et al*., 2015; Adeoye *et al*., 2016). Bacterial components in the tilapia GI seem to be similar both probiotic and without probiotic groups.

1.3.3.4 Hematological data

Basically, many hematological parameters as hemoglobin, hematocrit, hemoglobin, red blood cells, and protein content are used to assess the fish health status, and these data have reported to evaluate potential probiotics (Table 1.3). Different findings of blood parameters including red blood cells levels, hematocrit, hemoglobin, glucose and total protein of probiotic have been reported the potential of probiotic studies (Soltan and El-Laithy, 2008: El-Rhman *et al*., 2009). Positive effects of probiotics on blood parameters of high red blood cells, hematocrit, hemoglobin, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration more than the control

group have been reported by Eissa and Abou-ElGheit (2014). However, Standen *et al*. (2013) reported all blood parameters showed non-differences between probiotic and control groups.

1.3.3.5 Histological data

Epithelial cells and mucous tissues locate in the gastrointestinal tract are very important to the innate immune response. These cells have a function as a weapon immediately responses to against pathogens. The development of functions might be inducing by the GI microbe (Murray *et al.*, 1994; Gargiulo *et al*., 1998; Nayak, 2010). Then, the study of histological changes (microvilli cells, the epithelial layer thickness, the intra-epithelial leukocytes, mucous cells, and goblet cells, *etc*.) could be evaluated by using a light microscope and electron microscopes. The potential of probiotic has been provided to increase the epithelial layer thickness of the mid-gut (Nakandakare *et al*., 2013), to perform a massive number of an absorptive surface index (Ferguson *et al*., 2010; Standen *et al*., 2015), and microvilli cells of tilapia (Adeoye *et al*., 2016; Handan *et al*., 2016). Using probiotic at high dose can promote absorptive surfaces, intraepithelial leukocytes and goblet cells (Standen *et al*., 2016). Finally, fish fed probiotic groups were showed enhancement of the ability of phagocytes and reduced the intestinal damage cells, causing by a pathogen (Ngamkala *et al*., 2010).

Therefore, histological changes might be used to support the potential of probiotics and associated with gut immunes, fish health and growth performances.

1.3.3.6 Gut immunological data

The recognition of immune responses in aquatic fishes suddenly learns after hatching and contacts with water surrounding, which possibly contains chemicals, biochemical agents, or biological compounds (Tort *et al*., 2003; Galindo-Villegas and Hosokawa, 2004). The innate immune response is the primary defense mechanism consisting of the gut immunology (humoral parameters: complement system, antibacterial peptides and protease inhibitors), and cellular components (phagocytic leukocytes and non-specific cytotoxic cell). The adaptive immune response is later

acquired by the innate development. Both immune systems are synchronized together (Nayak, 2010).

The potential of probiotics has been reported to positively effect on blood cells, lymphocytes, monocytes and neutrophils (Aly *et al*., 2008b; Eissa and Abou-ElGheit, 2014; Standen *et al*., 2013). The other immune parameters have been evaluated by using some enzymatic activities as aspartate aminotransferase and alanine aminotransferase may cause damage to the GI tissues, however, the negative effect of probiotics on these parameters was found (Soltan and El-Laithy, 2008; El-Rhman *et al*., 2009).

The phagocytic estimation has found high expression in probiotic groups, which response to defense pathogens causing cell damages (Aly *et al*., 2008b&c; Wang et al., 2008). Probiotics have proved association with enzymatic releasing of myeloperoxidease to produce hydochorous acid killing pathogens and directly effect to microbial cell lysis (Wang et al., 2008). Some parameters of lysozyme activity, neutrophil adherence testing and serum bacterial activities have been demonstrated to be increasing in probiotic groups (Aly *et al*., 2008c; Nouh et al., 2009). Conversely, some immunological studies of lysozyme, total serum immunoglobulin, complement, specific-streptococcal antibody levels have been determined not different between the probiotic-feeding and without probiotic (Shelby *et al.*, 2006; El-Rhman *et al*., 2009).

1.3.3.7 Gene expression

Several studies reported that gene expression of cytokine families *(IL-1β*: interleukin-1 beta, *IL-2*: interleukin-2, *IL-10*: interleukin-10), *TGF- β*: transforming growth factor beta and *TNF-α*: tumor necrosis factor alpha) have related to the processing of the innate immune system and combined with microbial antigens or damaged cell occurrences (Reyes-Cerpa *et al*., 2013; Standen *et al*., 2016), while transferrin gene can express by pathogenic infection (Uribe *et al*., 2011). The *caspase-3* gene is indicated to apoptosis (cell death), while *PCNA* (proliferating cell nuclear antigen) is a signal of cell proliferation (Standen *et al*., 2016). In addition, HSP70 (heat shock protein 70) is used

to indicate the stressful condition, which benefit to maintain protein function, folding, and translocation (Iwama *et al*., 1999; Basu *et al*., 2002). These genes are used to evaluate the potential of probiotics.

Generally, bacterial infections and lipopolysaccharides stimulate the *IL-10* expression (Zhang, *et al*., 2009). Fish inflammations are caused by gram-negative bacteria, which may induce *TNF-α*, and *IL-1β* expressions (Savan and Sakai, 2006). The role of *TNF-α* gene typically plays varieties of host responses consisting of cell proliferation, differentiation, necrosis, and apoptosis, which might be induced by other cytokines. The *TGF-β* regulation as trans-forming growth factor may be expressed during the process of the cell development. He *et al*. (2013) reported that tilapia feed probiotic at a concentration of 10^9 cells g^{-1} diet displayed different expressions both up-regulated and down-regulated of these cytokine genes. Liu *et al*. (2013) found fish fed probiotic have expressed down-regulation of cytokine expression in the gut. Opposite with He *et al*. (2013) reported that probiotics might induce up-regulation of cytokine genes more than the control group. Similar reported by Standen *et al*., (2016) that up-regulations of cytokines ***(****TLR2****,*** *TNF*-α, *IL-1β, TGF- β* and *IL-10*) display in probiotic groups and increase of *caspase-3* (indicator of an apoptosis), and *PCNA* (indicator of cell proliferations).

Fish feed probiotic, which may decrease HSP70 expression (Avella *et al*., 2010). Generally, both pathogens and stressful conditions might activate up-regulation of HSP70 (Liu *et al*., 2012). Previous study, pathogenic infection can induce high HSP70 expression (Panakulchaiwit *et al*., 2008). Different doses of the probiotic-feeding have decreased HSP70 expression (He *et al*., 2013). However, the variation of HSP70 expressions in the intestine can display both up-regulation and down-regulation, which might depend on microbial changes in the gut of different times (Liu *et al*., 2013). Standen *et al*. (2016) reported that high expression of HSP70 was found in the probiotic group than the control group, which might relate to the change of the GI microbes.

1.3.3.8 Physiological changes

The stocking density of tilapia cultures could consider the optimal density to aware the agonistic behavior. Fish feed probiotics combining with different densities (3.7 and 40 $g.L^{-1}$) have shown an aggressive behavior at low density, while, high density shown the static behavior (Gonçalves *et al*., 2011). Agonistic activity shows no difference between the probiotic group and the control group; these fish were reared at 0.3 $g.L^{-1}$ of the stocking density, as reported by Soltan and El-Laithy (2008).

Probiotics may affect both external and internal effects. The external effect is related to growth phenotype while the internal effect is related to the digestive system, gene expressions, histological changes and immune systems. Probiotics for fish cultures may use a single dose or mixed doses, which can mix in fish feeds and added into the rearing system (Apún-Molina *et al*., 2009; El-Rhman *et al*., 2009; Cota-Gastléum *et al*., 2013). Moreover, probiotics can combine with the herb plant for supporting growth performances (Soltan and El-Laithy, 2008). Probiotic feed preparations may be favorable mixing before pelleted, after pelleted and after extruding processes (Nakandakare *et al*., 2013). Their potential effects of probiotics can be evaluated by using many parameters, which referred to the above descriptions.

1.4 Thesis aim and objectives

Research studies were based on standard methods for identifying and evaluating the potential of autochthonous bacteria as a novel probiotic for tilapia culture in Thailand (Chapter 2). Then, this thesis has proposed to examine a novel autochthonous probiotic for using in tilapia cultures, which are separated into two main objectives both *in vitro* and *in vivo* studies. These objectives linked together, are outlined in Figure 1.6.

Table 1.4 Experimental *in vivo* trials for evaluating potential probiotics for tilapia.

The initial weight (g)	Approxi-mately density (g/L)	No. fish/ unit (total fish)	System/ water capacity	Probiotics	Probiotic dose	Basal diets	Feeding technique (time per day; tpd)	Probiotic feed (weeks)	Parameter monitoring	Strains	References
(I) 0.013 (II) 0.03	(I) 0.005 (II) 0.006	(I) 23 (II) 13 (350)	Aquaria (57L)	Biomate SF-20 (*En. faecium*), Bioplus 2B (*B. subtilis*+ *B. licheniformis*), Bactocell PA10 MD (*Ped. acidilactici*) and Levucell SB 20 (*Sac. cerevisiae*)	10^{9-10} cfu.g^{-1} diet	50%CP (CF)	Ad libitum (2 tpd)	(a) ≈9.0 (b) ≈12.0	GP (WG), CT-SR ID, BD (TVC), and (BA, CA, LyC, TIg)	Nile tilapia	Shelby *et al*., 2006
0.14	0.01	66-67 (800)	Tank (1000L)	*Bacillus* sp. and LAB (autochthonous probiotics)	5×10^4 cfu.g^{-1} diet (LAB) and 10^3 cfu.ml^{-1} (*Bacillus* sp.)	45%CP (CF)	Ad libitum (?) Added in system every 15 days	≈19.0	GP (AG, AGR, SGR)	Tilapia	Apún-Molina *et al*., 2009
0.15	L: 0.08 H: 0.15	L: 10 H: 20 (600)	CRS (20L)	ALL-LAC™ (*Sac. faecium* + *Lac. acidophilus*; AllTech, Nicholasville, KY) & *Sac. cerevisiae* (BioSaf™, SafAgri, Minne-apolis, MN)	0.001 g.g^{-1} diet	27%CP and 40% CP	Ad libitum (4 tpd)	9.0	GP (ANU, AOMP, APD, CND, FCR, PER, SGR, WG) and SR	Nile tilapia	Lara-Flores *et al*., 2003
0.9	0.09	10 (420)	RWS (100L)	*Lac. brevis* and *Lac. acidophilus* (allochthonous probiotics)	$10^{5,7,9}$ cells.g^{-1} diet	42% CP	Ad libitum (2 tpd)	5.0	GP (FCR, WG), CT-SR, BD (DGGE) and GeE (*IL-1β*, HSP70, *TGF- β*, and *TNF-α*)	Hybrid tilapia	Liu *et al*., 2013
1.0	0.1	10 (240)	Tank (100L)	*B. subtilis* C-3102(CALSPORIN®, Calpis, Tokyo, Japan)	2.5 & 5 $\times10^5$ cfu.g^{-1} diet	36% CP	5% of BW (2 tpd)	8.0	GP (FCR, WG), BD (TVC, DGGE) and GeE (*IL-1β*, HSP70, *TGF- β*, *TNF-α*)	Hybrid tilapia	He et al., 2013
1.0	0.4	20 (420)	Aquaria (54L)	Premalac (*Lac. acidophilua*, *Bifedobacteria bifedum*, *Strep. Facecium*, torula yeast, Aspergillus oryzae extract, skim milk, vegetable oil and $CaCo_3$) and Biogen (allicin, enzymes, *B. subtilis*, ginseng extract)	0.001, 0.002 & 0.003 g.g^{-1} diet ≈10^7 cfu.g^{-1} diet	32% CP	4% of BW (3 tpd)	≈30.0	GP (ADE, EU, FCR, FPV, PUE, PPV, SGR), ID (WBC) and CT-SR	Nile tilapia	Ali *et al*., 2010
1.1	0.41	20 (540)	Aquaria (54L)	Premalac (*Lac. acidophilua*, *Bifedobacteria bifedum*, *Strep. Facecium*, torula yeast, Aspergillus oryzae extract, skim milk, vegetable oil and $CaCo_3$) and Biogen (allicin, enzymes, *B. subtilis*, ginseng extract)	0.002 g.g^{-1} diet	25, 25.5 and 30%CP	4% of BW (3 tpd)	≈17.1	GP (ADC, ADG, FCR, PER) and cost analysis	Nile tilapia	Ghazalah *et al*., 2010

Table 1.4 *Continued…*

The initial weight (g)	Approxi-mately density (g/L)	No. fish/ unit (total fish)	System/ water capacity	Probiotics	Probiotic dose	Basal diets	Feeding technique (time per day; tpd)	Probiotic feed (weeks)	Parameter monitoring	Strains	References
1.2	1.2	15 (90)	Aquaria (20L)	*Lac. acidophillus* (allochthonous probiotics)	10^6 cells.g^{-1} diet	24% CP (CF)	10% of BW (3 tpd)	≈2.1	GeE (*IL-1β* and TGe) and CT-SR	Tilapia	Villamil *et al*., 2014
1.3	0.02	12 (144)	Tank (600L)	LAB strains (autochthonous probiotics)*[A]	2.5 & 5 10^5 cfu.g^{-1} diet	45% CP (CF)	Ad libitum (?)	≈10.1	GP (SGR)	Tilpia	Cota-Gastléum *et al*., 2013
2.4	0.5	20 (240)	Aquaria (100L)	*Micrococcus luteus* and *Pseudomonas* sp. (autochthonous probiotics)	10^7 cells.g^{-1} diet	55% CP	3% of BW (2 tpd)	≈13.0	GP (FCR, NWG, PER, SGR), CT-SR, HD (Gl, Ht, Hb, RBC, TPr) and ID (BA, LyA)	Nile tilapia	El-Rhman *et al*., 2009
2.6	0.3	20 (420)	Aquaria (180L)	*B. subtilis* (allochthonous probiotics) and Biogen® (*Bacillus* spp.)*[B]	7x10^9 cells.g^{-1} diet	30% CP	10, 7 & 4% of BW (2 tpd)	≈13.0	GP (FCR, PER, SGR), HD (Ht, Hb), ID (LyA) and PMO (BO)	Nile tilapia	Soltan and El-Laithy (2008)
2.9	0.6	20 (240)	Aquaria (100L)	*Pseu. fluorescens* strains (autochthonous probiotics)	10^8 cells.g^{-1} diet	30% CP	3-5% of BW (2 tpd)	≈6.5	GP (BMG, MGR, SGR, WG), CT-SR, HD (Glo, Ht, Hb, RBC, TP) and ID (EnA, LeT, WBC)	Nile tilapia	Eissa and Abou-ElGheit, 2014
5.0	1.0	30 (960)	Aquaria (150L)	*Bacillus suntilis* (Sigma) and *Lac. acidophilus* (allochthonous probiotics)	10^7 cells.g^{-1} diet	Not reported	5% of BW (?)	8.0	GP (FCR, K, SGR), SR, CT-SR, ID (SBA) and HiD (LM-organs)	Nile tilapia	Nouh *et al*., 2009
5.2	6.5	25 (250)	Aquaria (20L)	PAS TR® (*Bacillus subtilis + B. toyoi*)	0.004 g.g^{-1} diet (4x10^8 cfu.g^{-1})	36% CP	1% of BW (3 tpd)	9.0	GP (AFC, DWG, FCR), SR and HiD (LM: EpH, EpT)	Nile tilapia	Nakandakare *et al*., 2013
5.2	1.0	30 (1920)	Aquaria (150L)	*B. subtilis* and *Lac. acidophillus* (allochthonous probiotics)	0.5 & 1x10^7 cells.g^{-1} diet	Not reported	5% of BW (?)	8.0	GP (FCR), CT-SR, BD, HD (Ht) and ID (BA, PhaA, LyA)	Tilapia	Aly *et al*., 2008c
6.5	0.1	80 (2880)	Cage (≈4800L)	*B.pumilus* (autochthonous probiotics) and Organic Green™	$10^{6\ \&\ 12}$ cells.g^{-1} diet	35%CP	3% of BW (2 tpd: summer) and 1% of BW (2 tpd: winter)	≈34.0	GP (Gr), CT-RLP, HD (Ht), and ID (PHaA, TLeC, LeT)	Nile tilapia	Aly *et al*., 2008b
6.8	0.8	30 (180)	RFW: Aquaria (250L)	*Enteroccus faecium* (allochthonous probiotics)	10^7 cfu.g^{-1} ml^{-1}	37%CP	3% of BW (3 tpd) Mixed in rearing system every 4 days	≈5.7	GP (DWG), HD (TP, TSP, Al, Gl, A/G) and ID (EnA, LyA, LyC, PhaA)	Tilapia	Wang *et al*., 2008
9.0	1.2	120 (600)**	Tank (900L)	*B. pumilus*, *B. firmus* and *Ci. Freundii* (autochthonous probiotics)	10^7 cells.g^{-1} diet	25%CP (CF)	5% of BW (3 tpd)	2.0	CT-SR	Nile tilapia	Aly *et al*., 2008a

Table 1.4 *Continued…*

The initial weight (g)	Approxi-mately density (g/L)	No. fish/ unit (total fish)	System/ water capacity	Probiotics	Probiotic dose	Basal diets	Feeding technique (time per day; tpd)	Probiotic feed (weeks)	Parameter monitoring	Strains	References
9.1	4.6	40 (320)	RWS (80L)	*P. acidilactici* MA 18/5 M (Bactocell®, Lammeemand Inc, Canada)	$2.8x10^6$ cfu.g^{-1} diet	44%CP	4% of BW (3 tpd)	6.0	GP (NWG, PER, k, HIS, SGR, VSI), BD (TVC), HiD (LM: AU, IEL's, GoC; TEM: MiL), HD (Ht, Hb, RBC, etc.), ID (WBC, LyC, LeT) and GeE (*TNF-α*)	Tilapia	Stenden *et al*., 2013
16.7	0.3	15 (240)	CRS (1000L)	*Bacillus* sp. (autochthonous probiotic) and *Enterococcus* (allochthonous probiotic)	$>10^6$ cells.g^{-1} diet	36% CP (CF)	8% of BW (3 tpd)	≈4.5	BD (QBT-FISH)	Tilapia	Del'Duca *et al*., 2013
(I) 12.3 (II) 12.7	L: 3.7 H: 40	L: 114), H: 109)	Tank (57L)	*Lac. rhamnosus* (allochthonous probiotics)	10^{10} cfu.g^{-1} diet	CF	3% of BW (?)	(I) 1 (II) 2	GP (PER, SGR, WG), HD (NCC), ID (CC, PO) and PMO-BO	Nile tilapia	Gonçalves *et al*., 2011
19.1	4.5	20 (180)	RWS (≈420L)	*B. amyloliquefaciens* (allochthonous probiotics)	10^8 cfu.g^{-1} diet	44% CP (CF)	4.5% and 3% BW (4 tpd)	≈14.0	GP (D, FCR, SGR), SR, BD (TVC), HD (Ht, Hb, RBC, TP) and ID (LeT, LyA, WBC)	Nile tilapia	Ridha and Azad (2012)
24.5	2.1	12 (144)	Aquaria (140L)	*Lac. plantarum* (autochthonous probiotics)	3.4 & $6.8x10^8$, $1.3x10^9$ cfu.g^{-1} diet	33-35% CP	3% BW (2 tpd)	≈5.7	GR (FCR, PER, PPV, SGR), CH-RPS, HD (Hb, RBC), ID (WBC, Tig, PhaA, LyA), GeE ((*IL-4*, *IL-12*, *IFN-γ*), HiD (TEM, SEM)	Nile tilapia	Hamdan *et al*., 2016
24.7	0.1	24 (240)	Concrete pond (≈8000L)	*Bacillus* spp. (Biogen®)	0.005, 0.01, 0.015 & 0.02 g.g^{-1} diet (≈10^7 cfu.g^{-1} diet)	30% CP	3% of BW (3 tpd)	≈18.0	GP (ER, FCR, PPV, SGR, WG) and Cost analysis	Nile tilapia	EL-Haroun et al., 2006
25	12.5	20 (900)	RWS (40L)	*B. subtilis*, *S. cerevisiae* and *A. oryzae* (Biogenic group, Brazil)	0.005 and 0.01 g^{-1} diet (10^9 cfu.g^{-1} diet)	28% CP	2% of BW (2 tpd)	6	GP (FCR, NGW), CT-SR, HD (Al, Gl. Glo, Hb, Ht, MCV, MCHC, TPr) and ID (CC, PhaA, TLeC, WBC)	Tilapia	Iwashita *et al*., 2015
29	9.7	50 (500)	RWS (150L)	Commercial probiotic, AquaStar® Growout (a mix of *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici*)	0.015, 0.03 g.g^{-1} diet	37-38%CP	1-5% of BW (4 tpd)	6	GP (FCR, PER, SGR), BD-DGGE, GeE (*caspase-3*, *PCNA*, HSP70, *TLR*2, *TGF-β*, *IL-10*,*TNF-α* and *IL-1 β*) and HiD (LM-AU, IET's, GoC)	Tilapia	Stenden *et al*., 2016

Table 1.4 *Continued…*

The initial weight (g)	Approxi-mately density (g/L)	No. fish/ unit (total fish)	System/ water capacity	Probiotics	Probiotic dose	Basal diets	Feeding technique (time per day; tpd)	Probiotic feed (weeks)	Parameter monitoring	Strains	References
33	L: 0.6 H: 2.0	L: 15, H: 50 (520)	RWS (800 L)	*B. subtilis* (strain C-3102-Calsporin®)	5×10^6 cfu.g^{-1} diet	34% CP	Ad libitum (3 tpd)	12	GP (Gr, FCR), SR, HD (Gl, Hb, Ht, RBC, MCH, MCHC, MCV) and ID (CC, IPha, PhaA, LeT, LyA, WBC)	Tilapia	Telli *et al*., 2014
35	2.1	30 (360)	RFW (508L)	Commercial probiotic (*containing B. subtilis, B. licheniformis and B. pumilus*; Sanolife PRO-F)	10^{10} cfu.g^{-1} diet	35% CP (CF)	3% of BW (3 tpd)	7	GP (FCR, PER, SGR, HIS, VSI), SR, BD (IPGS-HtSA), HD (Hb, Ht, MCV, MCH, MCHC, RBC), ID (LyC, TleC, WBC) and HiD (LM-AU, IEL's, Glo; SEM-EAA, ETAS, MCVT; TEM-MiL,MiD)	Nile tilapia	Adeoye *et al*., 2016
55	15	40 (320)	Tank (150L)	AquaStar® Growout (*Bacillus subtilis, Enterococcus faecium, Lactobacillus reuteri* and *Pediococcus acidilactici*: Biomin Holding GmbH, Austria)	0.005 g.g^{-1} diet	36% CP	1-3% of BW (4 tpd)	8	BD (TVC, BD-DGGE, IPGS-HtSA) and HiD (LM- AU, GoC, IEL's, Mul; SEM-ASI, MiD; TEM;MiL)	Tilapia	Standen *et al*., 2015
60-70	21.7	20** (60)	RWS (60 L)	*Lac. rhamnosus* (allochthonous probiotics)	$10^{8\&10}$ cfu.g^{-1} diet	CF	0.6% of BW (1 tpd)	2	ID (IHC, LeA, LyA and CA) and PMO-HP	Tilapia	Pirarat *et al*., 2006
70	?	30 (270)	Aquaria (?)	*Pseu. fluorescens* (autochthonous probiotics)	10^8 cells.g^{-1} diet	26% CP	2% of BW (2 tpd)	≈2.0	MR, HD (Al, Glo, Hb, RBC, TP,) and ID (LC, Let, WBC)	Nile tilapia	Eissa *et al*., 2014
111.0	37.0	40 (400)	RWS (120L)	Alchem Poseidon, Korea (*B. subtilis, Lac. acidophilus, Clos. butyricum* and *Sac. cerevisiae*)	$10^{7\text{-}8}$ cfu.g^{-1} diet	CF	1-2% of BW (2 tpd)	≈4.0	CT-SR, SST-MC, ID (BA, LyA, MC, PA, PhaA, OR) and HD (TP, Hg)	Tilapia	Taoka *et al*., 2006
150-180	58.0	20 (120)	RFW (61L)	*Lac. rhamnosus* (allochthonous probiotics)	10^{10} cfu.g^{-1} diet	CF	1% of BW (?)	≈2.0	MR, HiD (LM: MCN) and PMO	Nile tilapia	Ngamkala *et al*., 2010
175	26.3	12 (72)	RWS (80L)	*P. acidilactici* MA 18/5 M (Bactocell®, Lammeemand Inc, Canada)	10^7 cfu.g^{-1} diet	41-42% CP	1.5% of BW (3 tpd)	≈4.6	GP (FCR, PER, SGR) HD (He, Hb, TSeP), ID (LeT, LyA, PhaA) BD (TVC), IPGS) and HiD (LM: NL)	Red tilapia	Ferguson *et al*., 2010
185.0	5.6	3 (54)	Aquaria (100L)	*Lac. platarum* (autochthonous probiotics)	10^9 cfu.g^{-1} diet	32% CP	? (2 tpd)	≈2.1	HD (Hb, RBC), and ID (LeT, PhaA)	Nile tilapia	Dotta *et al*., 2011

*[A] Mixed with prebiotic; *[B] used herb/plant flower mixing; ** without replicates

CRS: closed recirculation system; RWS: recirculating water system; RFW: running fresh water/flow-through system; LS: Lentic system

GP: growth performance and others- AG: absolute growth; AGR: absolute growth rate; ADC: apparent digestion coefficient; ANU: apparent N utilization; APD: apparent protein digestibility; BMG: apparent organic matter and body mass rate; Gr: growth; CND: carcass N deposition; DWG: daily weight gain; D: density; ER: energy retention; EU: energy utilization; FCR: feed conversion ratio; FPV: fat productive value; HIS: hepatosomatic index; K: condition factor; NPU: net protein utilization; NWG: net weight gain; MGR: metabolic growth rate; PER: Protein efficient ratio; PPV: protein productive value; PUE: protein utilization efficiency; SGR: specific growth rate; VSI: viscerosomatic index;

SR: Survival rate after evaluating, and CH: after challenging with pathogen- *SST: salinity stress test; MR mortality rate; SR: survival rate; RLP: relative level of protection; RPS: relative percent survival*

BD: bacterial data- TVC: total viable count, QBT-FISH: quantify bacterial testing uses fluorescent in situ hybridization, GBDNA: genomic bacterial DNA, IPGS: identified probiotic by gene sequencing (DGGE: denaturing gradient gel electrophoresis, HtSA: high-throughput sequencing analysis)

HD: hematological data- Al/Glo ratio, Al: albumin, Glo: globulin, Gl: glucose, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume, MCH: mean corpuscular, hemoglobinHt: hematocrit, Hb: hemoglobin, NCC: nucleic acid concentration, Pl: plasma lipids, PC: protein content, RBC: red blood cells, TPr: total protein, TSeP: total serum protein

HiD: histological data- LM: light microscopy (AU: the intestinal perimeter ratio; arbitrary units, EpH: the height of the epithelial layer of the villi, EpT: thickness of the epithelial layer, GoC: goblet cells, IEL's: the number of intra epithelial leucocytes, NL: the number of leucocytes, MCN: Mucous cell number, MuL: mucosal fols lenght); TEM: transmission electrons microscopy (MiL: microvilli length, MiD: microvilli diameter); SEM: scanning electron microscopy (ASI: an absorptive surface area, ETAS: enterocyte total absorptive surface, EAA, enterocyte apical area, MCVT: microvilli count area)

ID: immunological data- BA: bactericidal activity, CA: complement activity, CC: cortisol concentration, EnA: enzyme activity, IHC: immunohistochemistry, IPha: index phagocytic, LeT: leucocyte types (%), LeA: leucocyte activity, LC: lymphocyte content**,** LyA: lysozyme activity, LyC: lysozyme content; serum lysozyme, OR: oxygen radicals, PhaA: phagocytic activity, PO: plasma osmolality, PA: protease activity, SBA: serum bactericidal activity, Tig: total immunoglobulin, TLeC: total leucocyte count, WBC: while blood cells (leukocyte)

GeE: gene expression- CEA: cytokine expression analysis, HSP70: heat shock protein gene, IL-1β: interleukin-1beta, TGe: transferrin gene, TGF- β: transforming growth factor beta, TNF-α: tumor necrosis factors

PMO: physiological and morphological data- BO: behavioral observation, MC: mucous changes, HP: histopathology (lesions**,** cell necrosis**,** cell structure)

The first objective was focused on *in vitro* trials. It begins by screening the GI bacteria of tilapia using conventional and molecular methods. These isolates were studied *in vitro* trials using multi-parameter as adhesion assays, auto-aggregations, antibiotic resistances, blood hemolytic assays, bile salt tolerances, pH tolerances, and temperature exposures (Chapter 3). Then, potential probiotics were used the Z-score method to select probiotic candidates using these parameters. The main hypothesis of this study was highly effective of probiotic candidates, which found in high scoring isolates.

Then, the investigation of probiotic selection was tested with the second objective to investigate *in vivo* trials both larval (Chapter 4) and juvenile tilapia (Chapter 5). These studies were monitored growth performances, probiotic monitoring in the GIT and intestinal histology (LM, SEM and TEM). Moreover, fish samples at the end of the trial were taken to induce extreme inductions, which were pathogenic and heat inductions.

Finally, the whole studies were generally discussed and summarized (Chapter 6), which included *in vitro* study and probiotic selection, the larval experiment and the grow-out experiment.

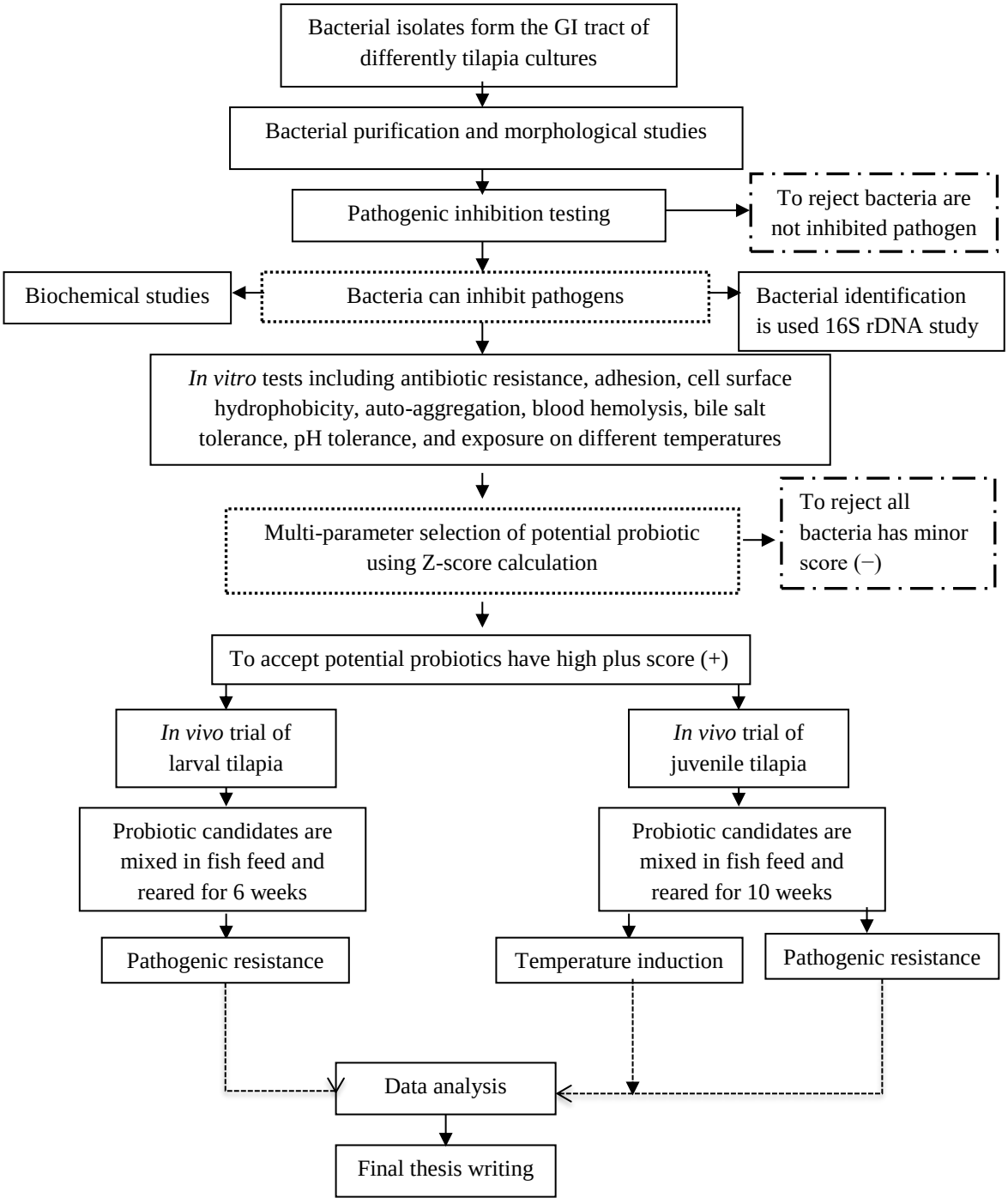


Figure 1.6 Flowed research protocols to evaluate autochthonous probiotic candidates for tilapia aquaculture in this study.

Chapter 2

General materials and methods

2.1 Introduction

In the present study, the intestinal bacterial from the tilapia GIT was isolated to evaluate the potential probiotics both *in vitro* (Chapter 3) and *in vivo* trials (Chapter 4 & 5), which were conducted by using the protocols described in this chapter. Other unique methods to evaluate multi-parameter of probiotic properties and selection are described in Chapter 3. Unless otherwise indicated, chemicals, reagents, and culture media were produced by Merck (Germany), Himedia (India), Sigma (USA), Qiagen (USA) and Bioline (USA). All experimental trials were conducted at King Mongkut's Institute of Technology Ladkrabang's (KMITL, Thailand) under Animals for Scientific Purposes Act and personal license U 1 - 07764 – 2561.

2.2 Fish dissections

In order to harvest tissue samples for analytical work fish were deprived of feed for 24 hours before dissections. Fish were euthanized with an overdose of tricaine methaesulphonate (MS-222, Sigma Aldrich Co, USA) to deep sedition and then the spinal cord was cut to minimize suffering. The intestine of these fish was removed under aseptic and cold conditions. The mid intestine was divided into three parts (Figure 2.1): part 1 for light microscopy (LM), part 2 for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (equally longitudinal section), and part 3 for probiotic monitoring or gene expression. The remaining GIT (part 4) was cut into

small pieces and crushed with a sterile pestle and mortar. This material was used to study microbial viable counts.

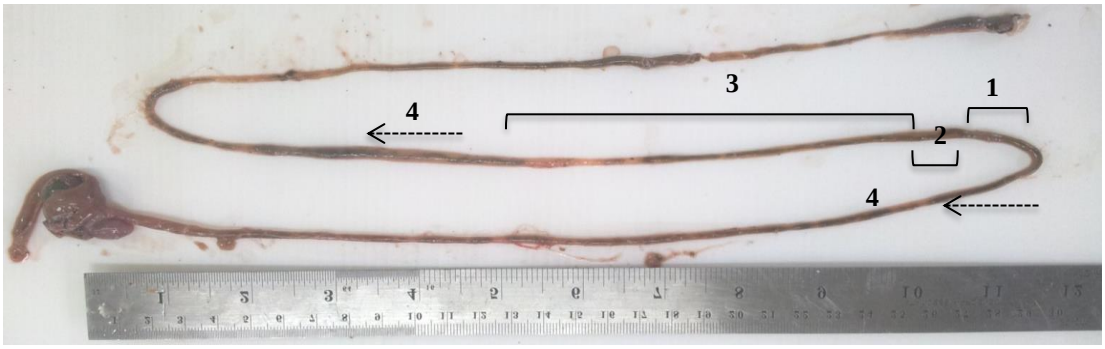


Figure 2.1 Regions of the intestinal tract of tilapia used in the experiments; part 1 for LM, part 2 for TEM and SEM, part 3 for probiotic monitoring or gene expression, and part 4 for microbial viable counts.

2.3 Microbial studies

2.3.1 Viable counts

The GIT of individual tilapia (Figure 2.1: part 4) was weighed and homogenized to perform viable counts by using serial dilutions and plate methods. Sterile saline (0.8% NaCl) was used as the diluent. The homogenized intestinal tract was diluted with sterile 0.8% NaCl (10^{-1}) and then put in a vortex mixer for 30 seconds. The homogenate was passed to sterile polyester filter (500µ) and the resulting solution was used to produce serial tenfold dilutions. Typically, 100 µL of 10^{-1}, $10^{-3\ to\ -4}$, $10^{-3\ to\ -4}$ and $10^{-7\ to\ -8}$ of diluted homogenate was used to spread on duplicate plates of de Man, Rogosa and Sharpe agar (MRS; Merck, Germany), tryptic soy agar (TSA; Merck, Germany) and nutrient agar (NA; Himedia, India), respectively. All plates were closed with elastic paraffin and kept in plastic bag. These plates were incubated at 25ºC for 48 hours. The cultivable bacterial

population in the GIT was determined by calculating the number of colony-forming units (cfu.g^{-1}). Duplicate or triplicate sets were undertaken per individual fish.

2.3.2 Bacterial purification and preservation

A single colony from each plate (section **2.3.1**) was selected to produce streak cultures on TSA plates, and then a single colony was selected to re-streak again. This process was repeated 5 times to ensure the bacterial purification and bacterial cells were than stained to confirm a similar Gram-phenotype (Figure 2.2). Finally, a single bacterial stock was established by stabbing a colony into TSA tubes incubating overnight at 30−32ºC, and then these were stored at 4ºC.

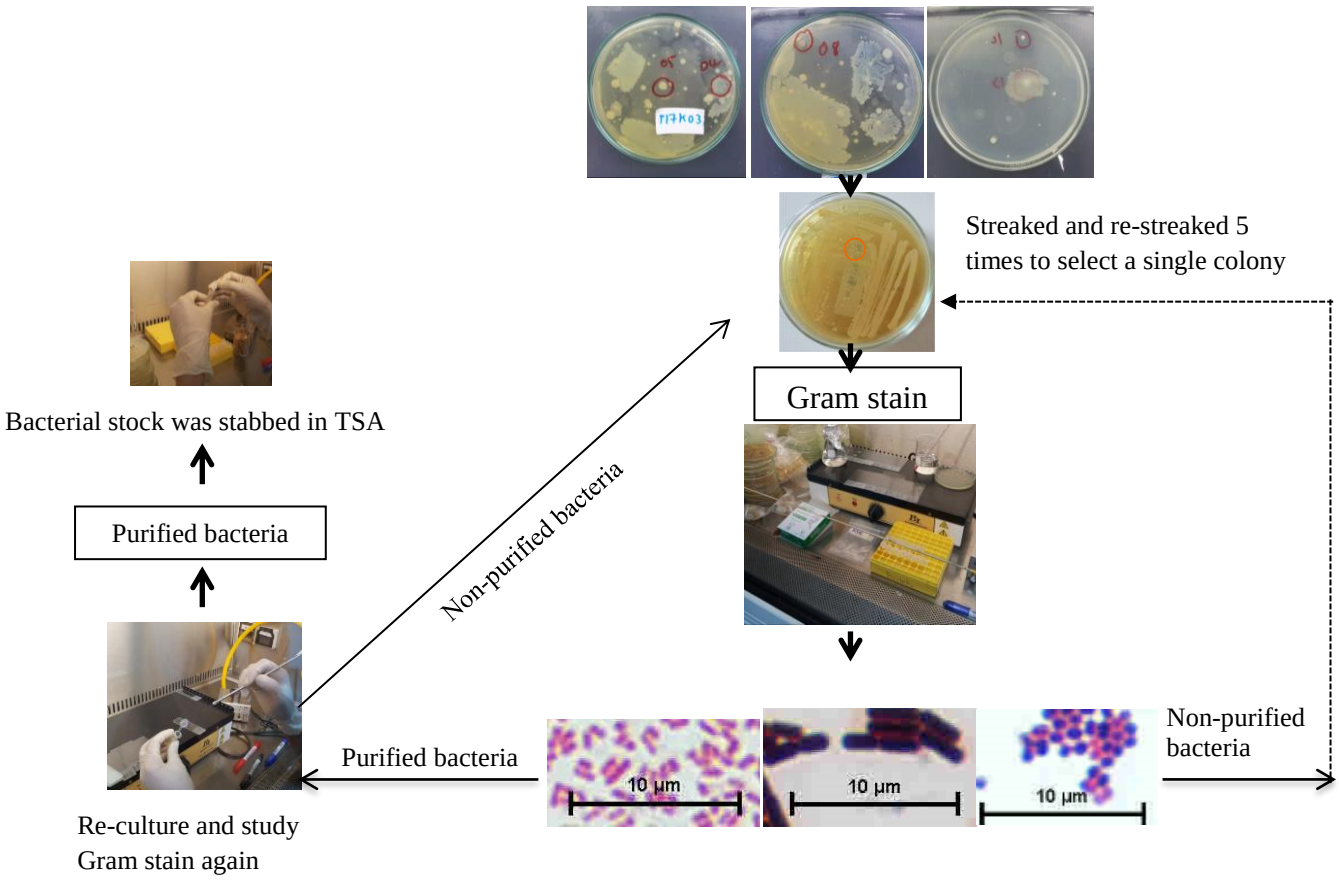


Figure 2.2 Protocol for bacterial isolation, purification and preserved stock.

2.3.3 Bacterial study

Typically, *Enterobacter* spp. were cultured on TSA plates and incubated overnight at 37^{o}C, while strains of bacilli (*Bacillus* sp. CHP02, RP01, and RP00) were cultured on selected *Bacillus* agar (BM, Himedia, India) (Figure 2.5: A−C) and the positive control probiotic *Pediococcus acidilactici* MA18/5M (Bactocell, Lallemand SAS) was cultured on MRS plates.

2.3.4 Sequence analysis of isolates

2.3.4.1 DNA extraction

Bacterial genomic DNA (section **2.3.3**) was extracted by using the traditional phenol/chloroform extraction method (Nishiguchi *et al*., 2002). In brief, two loops of bacterial cells were collected and transferred into 600 µl of sterile TE buffer and then homogenised on a vortex mixer for 10 seconds. Samples were centrifuged at 13,709 g for 10 minutes. The supernatant was mixed in 1000 µl of chloroform: iso-methyl-alcohol solution (24:1). These were centrifuged at 13,709 g for 10 minutes and the supernatant was transferred into 1,000 µl of cold 95% ethanol and stored at −20^{o}C for 24 hours. Then, these tubes were mixed and taken to centrifuge at 13,709 g for 5 minutes. The DNA pellet was then washed three times with 70 % ethanol. Finally, a total volume of 50 µl of TE buffer (pH 7.5) was used to re-suspend bacterial genomic DNA. DNA quantity was determined with an automated µDrop plate spectrophotometer (Thermo Scientific) and DNA concentration was estimated with the Skanlt® software. DNA extracts were kept at −20^{o}C until downstream processing.

2.3.4.2 Polymerase chain reaction (PCR)

Bacterial genomic DNA amplification was conducted by using the universal primers (27F: R'-AGAGTTTGATCCTGGCTCAG-3') and 1442R: (5'-GGTTACCTTGTTAGGACTT-3'). PCR tubes contained 12.5 µl of Genei Red Dye PCR Master mix (GeNei™, Merck), 2.5 µl of each primer (5 pmol), 2 µl of bacterial genomic DNA (***2.3.4.1***), and 10.5 µl of sterile distilled water. The thermal

cycling program (Labnet, Multi Gene II) was automatically controlled with the initial denaturation at 94^{o}C for 7 minutes, followed by 35 cycles at 94^{o}C for 30 seconds, 50^{o}C for 30 seconds, 72^{o}C for one minute, and the final extension at 72^{o}C for 5 minutes. The quality of PCR products in the agarose gel was observed under ultraviolet (UV) light and concentration of PCR products were assessed as described in section ***2.3.4.1***.

2.3.4.3 16S rDNA sequence analysis

The SpinPrep^{TM} Gel DNA Kit (Novagen) was used to extract genomic DNA from the band of PCR product, according to the manufacturer's instructions. 16S rDNA samples were sequencing by Macrogen Co. Ltd. (South Korea). Sequences were then submitted to a Basic Local Alignment Search Tool (BLAST; http://www.blast.ncbi.nlm.nih.gov) to identify bacterial species by using the similarity more than 99% to presumed taxonomic unit.

2.3.5 Probiotic monitoring in the intestine of tilapia

2.3.5.1 DNA extractions

The intestinal tract of fish (Figure 2.1: part 3) was used to monitor probiotic populations in the GIT. A commercial DNA extraction Kit (QIAamp DNA Stool Mini Kit, Qiagen) and a commercial reagent (TRIsure^{TM}, Bioline) were used for extracting bacterial genomic DNA.

2.3.5.1.1 DNA extraction using DNA kit: 200 mg of the homogenized GIT was added in 1.4 mL of ASL buffer, and mixed using the vortex mixer. Samples were incubated at 70^{o}C for 10 minutes. Then, these tubes were taken to centrifuge at 16,089 g for one minute before transferring the supernatant to a new tube. One Inhibit EX tablet was used to mix with this supernatant and then homogenized solution was centrifuged at 16, 089 g for 3 minutes. Later, 500 µL of supernatant was centrifuged again at 16,089 g for 3 minutes for removing 400 µL of supernatant to a new tube. Twenty µL of Proteinase K was added to the sample followed by 400 µL of AL buffer. These samples were incubated at 70^{o}C for 15 minutes, and 400 µL of absolute ethanol was then added.

Next, 600 µL of sample was transferred into a collection tube to centrifuge at 13,709 g for a minute. The solvent was then discarded and the samples were washed twice with AW1 buffer and AW2 buffer by ordering, which centrifuged at 16,809 g for 3 minute. Finally, DNA column was taken into a new tube and a volume of 50 µL of AE buffer was used to elute genomic bacteria. Bacterial DNA was kept at −20°C for the next study.

2.3.5.1.2 DNA extraction using a commercial reagent: approximately 50-100 mg of the homogenized intestinal tract was used to mix with 1,000 µL of Trisure reagent (Bioline). Samples were incubated at room temperature for 5 minutes and 200 µL of chloroform was added to mix in this tube, which was incubated at room temperature for 3 minutes. Samples were centrifuged at 16,809 g for 15 minutes (4°C) and supernatant was discarded. The volume of 300 mL cold absolute ethanol was used for tender mixing. Samples were incubated at room temperature for 3 minutes again. Then, samples were taken to centrifuge at 2,000 g at 4°C for 5 minutes and washed DNA pellets with 1,000 µL of 0.1 M Sodium citrate in 10% ethanol. These tubes were incubated at room temperature for 30 minutes followed by centrifugation at 2,000 g for 5 minutes (4°C). DNA pellets were washed with 1,500 µL of 75% ethanol and centrifuged at 2,000 g for 5 minutes (4°C). The supernatant was discarded to let DNA pellet dry. Finally, the DNA was re-suspended in 50 µL of TE buffer (pH 7.5). DNA quantity was determined with an automated µDrop plate spectrophotometer (Thermo Scientific) and DNA concentration was estimated with the Skanlt® software, and bacterial DNA was kept at −20°C for the next study.

2.3.5.2 PCR

PCR amplification was performed with specific probiotic primers in Table 2.1, which used Primer3 and BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These primers calculated the physical properties by using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) (personal contacted with Assoc. Prof. Srimek Chowpongpand). Trials samples were evaluated follow:

Bacillus spp. monitoring: the PCR mixture contained 12.5 µl of Go Taq ® Green Master buffer (Promega), 2.5 µl of 10 µM of *Bacillus* primers, 1 µl of DNA template and sterile distilled water was used to produce a final reaction volume of 25 µl. PCR amplification was carried out with an initial denaturation at 94 °C for 5 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute. The final extension was 72°C for 5 minutes. *Enterobactor* sp. monitoring: sample reactions consisted of 2.5 µl of 10 µM of FP47F primer and 2.5 µl of 10µM of FP47R, 1 µl of DNA sample, 12.5 µl Go Taq ® Green Master buffer (Promega) and sterile distilled water was used to produce a final reaction volume of 25 µl. PCR amplification was carried out with an initial denaturation at 94 °C for 3 minutes followed by 35 cycles at 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 1 minute, and a final extension step of 72°C for 5 minutes. *P. acidilactici* monitoring: sample reactions consisted of 12.5 µl of GoTaq® Green Master Mix, 2.5 µl of 10 µM of each primer (PaceF and PaceR), 1 µl of DNA template and sterile distilled water was used to produce a final reaction volume of 25 µl. PCR amplification was carried out with an initial denaturation at 94 °C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds and a final extension step of 72°C for 5 minutes.

2.3.5.3 Agarose gel electrophoresis

Agarose gels containing RedSafe DNA Stain (0.005 %) were used throughout the study at concentrations of 1.5% (w/v). A total volume of 5 µl PCR products of (section ***2.3.5.2***) containing 3 µl of 20x ultra power safe dye were run on through the agarose gels for 25 min at 100 V. Gels were photographed under UV light and recorded using a gel documentation system (Gene Flash). Finally, the gel document was interpreted to compare with standard DNA (5 µl of 1 kb DNA ladder containing 3 µl of 20x ultra power safe dye) and positive bands.

Table 2.1 Nucleotide sequences of probiotc primers used for monitoring probiotic levels in the GI tilapia

Primers	Bacteria	Primer sequences (5'-3')	Size (Mers)	Tm(°C)	Sizing of PCR products (pb)
FP45F	Probiotic *Bacillus* spp.	TTT TTG GTC TGT AAC TGA CGC TGA GGC	27	62	631
FP45R		ATC CGC GAT TAC TAG CGA TTC CAG C	25	62	
FP47F	Probiotic *Enterobactor* sp.	AGC CGC GGT AAT ACG GAG GGG T	22	65	554
FP47R		GTC TCA GAG TTC CCG AAG GCA CCA ATC	27	64.8	
PaceF	Probiotic *P. acidilactici*	TTT TAA CAC GAA GTG AGT GGC GGA CG	26	59.5	795
PaceR		GCG GAT TAC TTA ATG CGT TAG CTG CAG C	28	63	

2.4 Probiotics and fish feed trials

2.4.1 Probiotic preparation

Selected isolates (section **2.3.3**) were cultured in TSB overnight at 37 ^{0}C and then bacterial cultures were centrifuged at 2800 g for 15 min. Bacterial cell pellets were washed twice with sterile 0.85% NaCl and fresh probiotics were adjusted to make stock concentration at 10x in sterile 0.85% NaCl by using the optical density at 600 nm. These probiotic solutions were kept at 4^{o}C for mixing in basal fish feeds to produce experimental diets for use in chapters 4 and 5. A commercial probiotic *Pediococcus acidilactici* MA18/5M (Bactocell, Lallemand SAS) was cultured in MRS. The final concentration of a commercial probiotic was adjusted to produce a 10x stock solution in sterile 0.85% NaCl by using the optical density at 600 nm according to Ferguson *et al*., (2010), with some modifications.

2.4.2 Fish feed and preparation of probiotic feeding

Three commercial feeds were used as the basal diets (Figure 2.3 & Table 2.3) for the *in vivo* feeding trials in Chapters 4 and 5. The first feed was in a fine form with sizing less than 1 mm (CP 9000 diet from CP Co., Ltd., Thailand; containing 40% of crude protein, 6% of total fat and 3% of ash) and was used in the first half (day 0 to end of third week) of the larval trial in Chapter 4. The second feed was in a crushed form with sizing between 1.0 to 1.5 mm (CP 9001 diet from CP Co., Ltd., Thailand; containing 38% of crude protein, 5% of total fat, and 3% of ash), and was used in the second half (End of week 3 until the end of week 6) of the larval trial in Chapter 4). The third feed was in a pellet form with sizing 4.0 mm of diameter (Prema diet from Premafeed Co., Ltd., Thailand: 30% of crude protein, 3% of total fat, 6% of crude fiber, 12% of ash, 37 of % NFE and 3,350 Kcal/kg); this feed was used in *in vivo* juvenile trial (Chapter 5).

A **B** **C**

Figure 2.3 Different forms of commercial feeds, A: fine form used in the initial larval rearing (Chapter 4), B: crushed form used at 3 weeks to the end of the larval trial (Chapter 4), and C: pellet form used in juvenile trial (Chapter 5).

All three basal feeds were used to produce six treatment diets are described in Table 2.2. These diets were prepared by using 200 mL of probiotic stock (section **2.4.1**) to mix with 1000 g of basal feed, which was then dried at 40−45 ^{0}C for 6−10 hours. The control group (T6) was produced by adding 200 mL of sterile 0.85% NaCl with 1000 g of the basal diet. During feed incubations, the weight before and after incubations was accepted 0.1% of different weight. Fresh diets were prepared on a weekly basis.

Table 2.2 Experimental groups in *in vivo* trials (Chapter 4 & 5)

Groups	**Probiotic dose (cfu.g^{-1} diets)**	**Abbreviated groups of *in vivo* trials**
A commercial feed + *Bacillus* sp. CHP02	$6-7\times10^{6}$	T1
A commercial feed + *B. aryabhattai* RP01	$2-4\times10^{6}$	T2
A commercial feed + *B. megaterium* RP00	$1-2\times10^{6}$	T3
A commercial feed + *Enterobacter* sp. NP02	$5-8\times10^{7}$	T4
A commercial feed + *P. acidilactici*	$9-10^{7}$	T5
A commercial feed	−	T6

The nutritional compositions as dry matter was estimated by using temperature at 85°C for constant drying, crude protein with a micro-Kjeldahl apparatus, crude lipid with Soxhlet extraction, and ash with a muffle furnace of the experimental diets were estimated using proximate analysis according to AOAC (1997). Different fish feeds after adding probiotics were found the moisture content ranging from 6.5 to 7.5 of the first feed, 6.7 to 6.9 of the second feed and 7.7 to 8.7 of the third feed. These were shown nutritional compositions in Table 2.3.

Table 2.3 Percentage of nutritional compositions of experimental groups after adding different probiotics for *in vivo* trials.

Groups	The first feed			The second feed			The third feed		
	Crude protein	Lipid	Ash	Crude protein	Lipid	Ash	Crude protein	Lipid	Ash
T1	42.6±0.32	4.9±0.19	12.5±0.04	41.2±0.21	5.0±0.25	12.8±0.19	41.2±0.21	5.0±0.25	12.8±0.19
T2	42.7±0.07	5.0±0.00	12.5±0.11	41.1±0.85	5.0±0.27	12.8±0.11	41.1±0.85	5.0±0.27	12.8±0.11
T3	42.1±0.76	4.9±0.09	12.5±0.14	41.5±0.23	5.0±0.46	12.9±0.11	41.5±0.23	5.0±0.46	12.9±0.11
T4	42.0±0.16	4.9±0.60	12.5±0.12	41.7±0.17	5.1±0.32	12.8±0.00	41.7±0.17	5.1±0.32	12.8±0.00
T5	42.3±0.24	5.1±0.02	12.5±0.07	41.3±0.12	4.8±0.11	12.7±0.02	41.3±0.12	4.8±0.11	12.7±0.02
T6	42.6±0.37	5.1±0.23	12.5±0.17	41.4±0.18	4.8±0.07	12.8±0.19	41.4±0.18	4.8±0.07	12.8±0.19

2.5 Growth parameters

The weight and total length of tilapia in the growth trials were monitored weekly after a feed deprivation period of 24 hours.

Larval fish (Chapter 4) were randomized into a small container with having paper tissue moister and a standard scale. Then, fish samples were recorded total weight and total length. At the end (week 6) of the larval trial (Chapter 4), fish were individually weighed and measured. Fish samples in Chapter 5 were individually recorded by using microchip identification. The microchip (8 mm long × 1 mm diameter, low−frequency around 134.2 kHz which refer to ISO11784/11785 animal ID transponder FDX−B) was injected into the ventral cavity of juvenile tilapia (3-4 g of weight) for individual recording. These fish were acclimated for three weeks to allow the epidermis to heal post injection before undertaking the feeding trial (Meeanan *et al*., 2009). Experimental fish was automatically recorded the total weight and total length of individual fish by using Retina System, (Matcha IT, Thailand; http://majchait.wixsite.com/majchait: Figure 2.4).

Figure 2.4 The automatic recording system (Matcha IT, Thailand) was used to monitor individual tilapia growth

2.5.1 Parameter estimations

Data recording was used to calculate the following: average wet weight (g), average total length gain (TLG, %), average of increasing weight (IW: g.week^{-1}), average weight gain (WG, %), average total length (TL: cm), specific growth rate (SGR, %.day^{-1}), average daily growth (ADG, g.day^{-1}), Fulton's condition factor (K), feed conversion ratio (FCR) and the relative intestinal length (RIL). These data were analyzed by using the following formulae:

$$TLG\ (\%) = 100 \times \frac{(TL_T - TL_{T0})}{TL_{T0}} \quad \ldots(1)$$

$$IW\ (\%) = Total\ weight_{week_n} - Total\ weight_{week_{n-1}} \quad \ldots(2)$$

$$WG\ (\%) = 100 \times \frac{(W_T - W_{T0})}{W_{T0}} \quad \ldots(3)$$

$$TLG\ (\%) = 100 \times \frac{(TL_T - TL_{T0})}{TL_{T0}}$$

$$SGR\ (\%, per\ day) = 100 \times \left[\frac{LnW_T - LnW_{T0}}{T}\right] \quad \ldots(4)$$

$$ADG\ = \left[\frac{W_T - W_{T0}}{T}\right] \quad \ldots(5)$$

$$K = \frac{(100 x W_T)}{(TL_T^3)} \quad \ldots(6)$$

$$FCR = \frac{(Total\ feed\ intake_T - Total\ feed\ residue_T)}{(W_T + Total\ weight\ of\ dead\ fish_T - Wt_{T0})} \quad \ldots(7)$$

$$RIL = \frac{(total\ length\ of\ the\ GIT)}{(TL)} \quad \ldots(8)$$

Where W = wet weight (g), TL = total length (cm), T_0 = the initial time of the trial, T =duration of feeding (days).

2.5.2 Survival rate

The percentage of survival rate (SR, %) was reported after finishing trial. The survival rate was defined as the ratio of the total number of fish at the initial to the total number of fish at the end of the trial as follows:

$$SR\ (\%) = 100 \times \frac{number\ of\ the\ final\ fish}{number\ of\ the\ initial\ fish} \quad \ldots(8)$$

2.5.3 Histological studies of the intestinal tract

2.5.3.1 Light microscopy (LM)

Small samples (Chapter 4) of the GIT (Figure 2.1: part 1) were placed between sponges within cassettes while large samples (Chapter 5) were placed in a cassette without a sponge (Mumford 2004). These samples were preserved in 10% buffered formalin. Sample cassettes were placed into the tissue processor (Leica, TR 1020). The program was set to immerse in each container of different percentages of graded alcohol (50, 70, 80 and 95%), three containers of absolute alcohol, and two containers of melted paraffin; this program emerged the samples in each container for 1 hour. Samples in paraffin blocks were prepared, trimmed and then cut with a semi-automatic microtome (Microm, Germany) to produce 5 μm sections transverse cross sections of the intestine. Sections were stained with hematoxylin and eosin (H&E) regarding following Mumford (2004). Stained slides were mounted on a permanent medium under a glass coverslip.

Triplicate samples of each replicate in treatment were recorded intestinal photographs to count the goblet cell density (cell/0.1mm^2) by using the NIS-Elements D 3.2 Ink software in a PC computer, which has the Nikon's digital sight DS-U3 interfacing the camera in a compound light microscope (20-40X: Olympus BX51).

2.5.3.2 Transmission electron microscopy (TEM)

Samples (Figure 2.1: part 2) were processed according to Schneider (2014) with some modifications. Samples were cleaned with phosphate-buffered saline (PBS; pH 7.3) twice before maintaining in 2.5% glutaraldehyde at 4^{o}C. Samples were cleaned in cold 0.1 M Na-cacodylate buffer (pH 7.2) three times. Then, samples were fixed in 1% osmium tetroxide (OsO_4) in darkness for 1 hour, and removed to clean in Na-cacodylate buffer (pH 7.2). These samples were dehydrated with different percentages of ethanol series following as follows: 30% (30 minutes), 50% (30 minutes), 70% (30 minutes), 80% (30 minutes), 90% (30 minutes), 95% (30 minutes), and absolute

ethanol (3 times: 30 minutes each). Samples were then processed with infiltration of different concentrations of resin (LR white resin, Sigma) as follows: 30% resin (24 hours), 50% resin (5 hours), 70% resin (5 hours), and 100% resin (24 hours). Finally, accelerator (1% v/v) was used to mix in absolute resin, and 580 µl of this solution was pipetted into beem capsules and the samples were then placed in the beem capsule. Capsules were placed at room temperature for resin polymerization. Samples were trimmed and cut into semi-thin sections using a diamond knife (DiATOME). Samples (0.5 um) were picked up into drop water on the glass slide and dried on the hot plate (90^{o}C) to stain with methylene blue, and then initially screened by a light microscope. The position on block was marked for cutting. The ultrathin section of selected block (≈90 nm) was placed on copper grids, and stained with saturated uranyl for 30 minutes. These were rinsed with distilled water and stained with Reynolds lead citrate (Lewis and Knight, 1977) for 30 minutes. Finally, samples were recorded by using a TEM (Phillips: Techni20, Holland) for using measurement of microvilli lengths (h_{mi}) and microvilli widths (w_{mi}) of these micrographs (Figure 2.5).

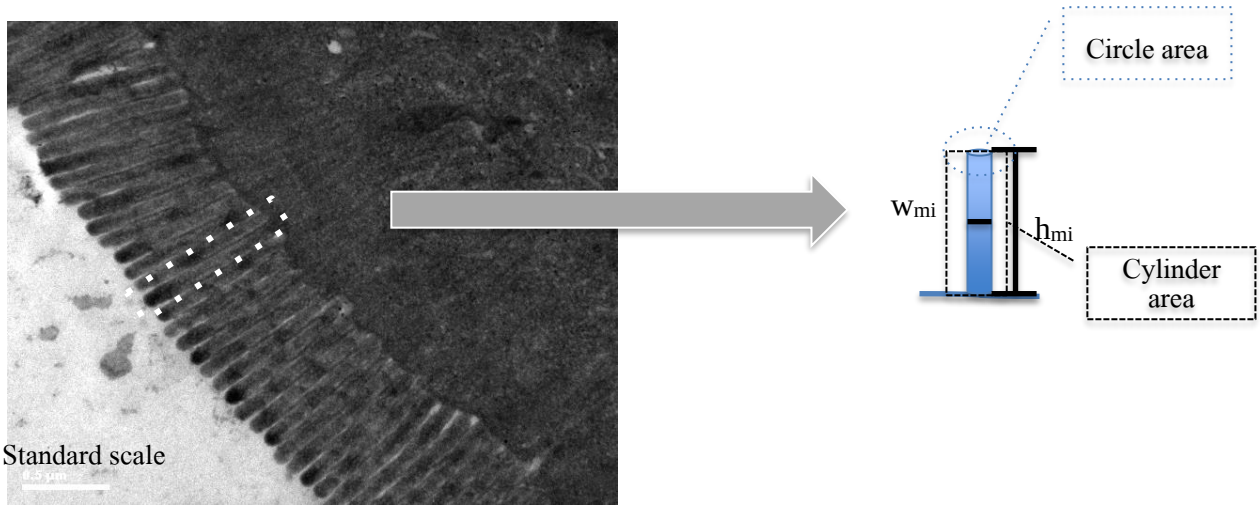


Figure 2.5 Microvilli area measurements

2.5.3.3 Scanning electron microscopy (SEM)

Samples were prepared according to Schneider (2014) with some modifications. The pieces of the GIT (Figure 2.1: part 2) were cleaned with phosphate-buffered saline (PBS; pH 7.3) for twice and then these samples were washed in 1% S-carboxymethyl-L-cysteine for a minute to dissolve mucus before transferring to 2.5% glutaraldehyde. Samples were cleaned and dehydrated with different percentages of ethanol series following as follows: 30% (30 minutes), 50% (30 minutes), 70% (30 minutes), 80% (30 minutes), 90% (30 minutes), 95% (30 minutes), and absolute ethanol (3 times: 30 minutes each). Samples were then critically point dried (SPA 400). Dried samples were transferred onto aluminum stubs for coating gold (Cressington Sputter Coater, 108 auto). Samples were then screened (Carl Zeiss: EVO® HD SEM, USA) to record micrographs of microbial colonization of the intestine.

2.6 Statistical analysis

Data analysis began by testing normal distribution and then calculating depended on the experimental design. The findings were displayed in terms of mean ± standard deviation. A significant difference between groups was accepted for $P < 0.05$. Some parameter's data were transformed to calculate analysis of variance (ANOVA). These data were analyzed using the Systat software ver. 5.02 (Illinois, USA).

Chapter 3

In vitro assays for selecting the potential probiotics

3.1 Abstract

Thirty-four microbial colonies were isolated from the intestine of tilapia (n=19), which cultured from differed sources. Fifteen isolates displayed inhibition of pathoginic bacteria (*A. hydrophila* or/and *S. iniae*). These bacteria were identified as *B. cereus* CHP00, *B. cereus* NP00, *B. cereus* NP01, *Bacillus* sp. RP00, *Mac. caseolyticus* CHP03, *Stap. arlettae* CHP04, *Stap. sciuri* NP04, *Bacillus* sp. RP01, *Bacillus* sp. CHP01, *Bacillus* sp. CHP02, *Bacillus* sp. RC00, *Bacillus* sp. RC01, *Bacillus* sp. RC02, *Enterbactor* sp. NP03, and *Enterbactor* sp. NP02. These bacteria were then carried out to evaluate potential probiotic *in vitro* trials by using multi-parameter: antagonistic activity, cell-adhesive potentials, hemolytic activities, antibiotic resistance, pH and bile salt tolerances and specific growth rates. The results of cell-adhesive potentials and specific growth rates between isolates were shown different significances ($P \leq 0.05$). Seven of fifteen isolates (*Bacillus* sp. RP00, *Bacillus* sp. RP01, *Bacillus* sp. RC00, *Bacillus* sp. RC01, *Bacillus* sp. CHP02, *Mac. caseolyticus* CHP03 and *Stap. sciuri* NP04) were shown acceptable to twelve antibiotics tested, and five isolates: *B. cereus* CHP00, NP00, and NP01 and *Bacillus* spp. CHP01 and RC02 were positive effect on haemolytic activities. All isolates were resistant to 6% bile salts condition and all *Bacillus* strains were able to tolerate pH 2. The findings were then combined and sigma scores (Z−score) were used for ranking the most promising isolates. The top three ranking candidates after using the Z−score for calculation were found to be *Bacillus* sp. CHP02 (Z=1.14), *Bacillus* sp. RP01 (Z=1.09) and *Bacillus* sp. RP00 (Z=0.94). These probiotic candidates were then selected for evaluation in the next *in vivo* trials.

3.2 Introduction

Probiotic properties have been reported using many parameters in *in vitro* trials, such as safe use (antibiotic resistance and hemolysis activity), probiotic characterizations as a resist to gastric acidity and bile acid, adherence, and pathogenic antagonism (Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Gaggìa *et al*., 2010; Merrifield *et al*., 2010; and Chemlal-Kherraz *et al*., 2012), which assessed to select potential probiotics as the classical method in *in vitro* trials.

Different techniques, parameters and bacterial isolates have been carried out to select potential probiotics (Aly *et al*., 2008; El-Rhman *et al*., 2009; Chantharasophon *et al*., 2011; Chemlal-Kherraz *et al*., 2012; Gobinath and Ramanibai, 2012; Del'Duca, 2013; Muñoz-Atienza *et al*., 2013). These have produced many findings including pathogen inhibition, blood hemolysis, susceptibility to antibiotics, ability to produce lactic acid and pH, bile salt tolerances, mucin degradation and enzymatic activities, which used to support the selection of potential isolates. A key question is how to combine different parameters by using a systematic calculation to elucidate high potential qualities of selected probiotics. In this study, the protocol to select high potential of probiotic candidates was improved by using the standard normal distribution (Z−score) as a classical method (Best and Kahn, 1998; Gordon, 2006). This method combined the results of multi-parameter by calculating standard deviations of each parameter from their means. These results were ranked by isolate-scores, which assumed high scoring isolates as highly effective of probiotic candidates.

The objectives of this study were to isolate, characterize and identify the autochthonous bacteria from the intestinal of tilapia, determine their potentials of probiotic properties (multi-parameter) such as adherence with the intestinal epithelial cells of tilapia, adhesion to hydrocarbons, auto-aggregation, antibiotic resistance, blood hemolysis, bile salt and acid tolerances, and temperature

exposures in *in vitro* trials. Finally the Z−score was proposed to select probiotic candidates as combined selection.

3.3 Materials and Methods

3.3.1 Bacterial isolation

3.3.1.1 Tilapia samples

Tilapia were located from different sources such as closed system (Nile tilapia KMITL strain: group 1 and Nile tilapia Chitralada strain: group 2), an earthen pond (Nile tilapia: group 3 and red tilapia: group 4), and a cage culture (red tilapia: group 5). They were acclimatized in the closed recirculating system for four weeks. This system has 760 l of capacity and filled with freshwater to constant level. The flow rate was adjusted to 10 $l.min^{-1}$. During the acclimation period, water qualities in the system were 2.3−3.4 $mg.l^{-1}$ (DO), 27−29ºC (water temperature), and 7.56−8.24 (pH). These fish were fed once daily with a commercial fish feed (Inteqc Feed, no.461).

3.3.1.2 Bacterial isolation and purification

Fish samples were starved for two days and then individually killed as detailed in section **2.2**. These fish (n=19) ranged from 4 to 288 g in weight, 7 to 26 cm of total length, 5 to 21 cm of body length, 34 to 172 cm of intestinal length, and 0.17 to 5.65 g of the intestinal weight, which used the GIT to isolate bacteria. The viable counts were studied as described in section **2.3.1** by using pour plates. Finally, photographs of agar plates were recorded and calculated cfu of each plate by using manual calculation of ImageJ 1.48 software.

A single colony was screened from each plate and then purified to preserve for next study as followed in section **2.3.3**. In addition, bacterial Gram-stain phenotypes of isolates were recorded

photographs by using the NIS-Elements D 3.2 Ink software in a PC computer, which has the Nikon's digital sight DS-U3 interfacing the camera in a compound light microscope (20-40X: Olympus BX51).

3.3.2 Pathogenic bacterial inhibition

3.3.2.1 Bacterial pathogenic preparations

In this study, bacterial pathogens *A. hydrophila* and *S. iniae* were supplied by the Inland Aquatic Animal Health Research Institute (AAHRI), Thailand. Bacterial virulence was activated for two times by injecting 100−300 µl (10^7 cells.ml^{-1}) of fresh cells into the dorsal muscle of healthy Nile tilapia (weight 20 to 30 g). Samples were reared in glass containers with aeration to observe pathogenic infections for three days. The pathogenic symptoms include skin lesions for *A. hydrophila* and erratic swimming behavior for *S. iniae*. A sterile loop was used to scrape skin lesions of diseased fish caused from *A. hydrophila* to streak on TSA plates, and liquid behind the eye of *S. iniae* diseased fish was used to streak on TSA plates. All plates were incubated at 32ºC for 24 hours and then the process was repeated to activate again. Prior to upscaling to prepare inoculating solutions, a single colony of the pathogenic bacterium was confirmed species identification by using Gram-stain and API20 kit (Biomérieux).

3.3.2.2 Antagonistic screening

The antagonistic protocol was performed according to Vine *et al*. (2004) with some modifications. In brief, fresh bacterial pathogens (section ***3.3.2.1***) were spread on TSA plates and incubated at 32ºC for 2 hours and bacterial isolates (prepared as **2.3.3**) were then spotted in these agar plates. Plates were incubated at 32ºC for 20 hours and observed clear zones around spot cultures.

3.3.3 Phenotypic characterizations

Bacterial isolates displaying inhibition to pathogenic bacteria (section ***3.3.2.2***) were studied phenotypic traits such as carbohydrate fermentation, triple sugar iron, methyl red, Voges-Proskauer, citrate utilization, oxidation-fermentation, oxidase, catalase, decarboxylase, indole, motility, granule staining, endospore and capsule forming (Prescott, 2002; Collins *et al*., 2004).

3.3.4 16S rDNA identification

Bacterial isolates were cultured as **2.3.3**, and bacterial DNA were extracted following ***2.3.4.1***. These genomic extractions were amplified by using universal primers (section ***2.3.4.2***). PCR products were studied by using agarose gel electrophoresis (section ***2.3.4.3***). Finally, DNA sequencing of bacterial isolates were submitted to presume species identification with reference in GenBank (section ***2.3.4.4***) by using the similarity more than 99% to presumed taxonomic unit.

3.3.5 *In vitro* trials

3.3.5.1 Adherence assay to the tilapia intestinal cells

Tilapia intestinal cells were collected according to Balcázar *et al*., (2007) and Grześkowiak *et al*., (2012). In brief, five healthy tilapia were sacrificed with an overdose (MS222) for removing the intestinal tract under aseptic conditions. The sterile loop was used to scrape the epithelial cells of the mid-gut intestine and transferred into a sterile plate with containing PBS (pH 7.5) and then, the epithelial cell solution was filtered through an autoclaved filter (500μm). Cell samples were centrifuged at 16,089 g for 10 minutes and twice washed using PBS (pH 7.5). Finally, the cell density was adjusted to approximate 0.02 of the optical density (OD_{600}).

The initial study, 5 ml of each bacterial preparation (9×10^{8} cells.ml^{-1}) was mixed with 5 ml of the epithelial cell solution. The total volume of 1.2 ml of this suspension was transferred into the Eppendorf tube with totally 8 tubes and allowed adhesion at room temperature (25^{o}C). Duplicate

tubes of the samples at the incubation times in 0, 2, 4, and 6 hours were recorded the absorbance (OD_{600}). A dye exclusion test (the trypan blue) was used to monitor bacterial adhesion to epithelial cells (Longo-Sorbello *et al*., 2006).

3.3.5.2 Adhesion to hydrocarbon solvents

Bacterial adhesion to hydrocarbon solvents was examined according to Rosenberg and Rosenberg, (1985), Kos *et al*., (2003), Collado *et al*., (2008) and Grześkowiak *et al*., (2012) with some modifications. Bacterial cells in PBS (pH=7.5) were adjusted to approximately 1 (OD_{600}). A volume of 1.5 ml of cell suspension was transferred to gently mix with 1.5 ml of chloroform for 30 seconds and incubated at room temperature (25^{o}C). The initial exposure (A_0) of the samples in duplicates was recorded the absorbance (OD_{600}). After incubation for 30 minutes, the aqueous phase in the upper solution of duplicates of each isolate was transferred to measure the absorbance (A_1: OD_{600}). In order to determine adhesion in hexane was performed as the same chloroform, however the aqueous phase at the bottom tube was used to measure the absorbance (OD_{600}).

3.3.5.3 Auto-aggregation assays

Auto-aggregation assays in both PBS and sterile 0.85% NaCl were performed according to Collado *et al*., (2008) and Grześkowiak *et al*., (2012), with some modifications. At the initial assay, stock cell concentrations in PBS (pH 7.5) of bacterial testing were adjusted to approximately 1 at OD_{600}. A volume of 100μL cell suspension of each isolate was transferred into the Eppendorf tubes in the duplicates and allowed to adhere for 0, 2, 4, and 6 hours. After incubation in these times, a total volume of 900 μl PBS (pH 7.5) was used to mix with each tube and then recorded the absorbance (OD_{600}). Auto-aggregation assay using sterile 0.85% NaCl, approximately 1 at OD_{600} of bacterial cell density in sterile 0.85% NaCl was prepared and the protocol was performed at the same of PBS.

3.3.5.4 Antibiotic susceptibility test

Antibiotic susceptibility test using the disk diffusion method was evaluated according to Bauer *et al*. (1966) with some modifications. In brief, a volume of 100 µl (9×10^8 cells.ml^{-1}) of fresh bacterial preparations were spread on TSA plates and dried in the laminar flow cabinet for 45-60 minutes. Twelve commercial antibiotic discs (Oxiod, UK) having ampicillin 10 µg (AMP 10), cephalothin 30 µg (KF 30), enrofloxacin 5 µg (ENR 5), erythromycin 15 µg (E 15), gentamycin 10 µg (CN 10), kanamycline 30 µg (K 30), neomycin 30 µg (N 30), nitrofurantoin 300 µg (F 300), oxolinic acid 2 µg (QA 2), oxytetracycline 30 µg (OT 30), sulphamethoxazole/thrimethoprim 25 µg (SXT 25), and tetracycline 30 µg (TE 30) were added to the plate in the duplicate discs. These plates were incubated at 32^o C for 24 hours. The apparent of clear zone around antibiotic discs was measured a diameter (mm) and interpreted to susceptible (S), intermediate (I), or resistant (R).

3.3.5.5 Hemolytic activities

Sheep blood (MDX1407077) and tilapia blood were used to determine hemolytic activities of samples according to Apún-Molina *et al*., (2009) and Nayak and Mukherjee (2011) with some modifications. A 1 ml syringe containing heparin was used to take blood samples form ten healthy tilapia (averaged 400 g) and then mixed with autoclaved blood agar (5%v/v in Brain heart infusion agar; HIMEDIA, India). These blood agar plates were sterilized with UV in the laminar flow cabinet for 45-60 minutes. Four wells having 0.6 cm diameter were made in these agar plates, and then 20 µl of fresh bacterial preparation (9×10^8 cells.ml^{-1}) was transferred into duplicate wells. These plates were incubated overnight at 32^{o}C under aerobic conditions. Hemolytic activities were observed by using apparent clear zone around wells and these diameters were recorded. Moreover, visualizations of blood hemolysis were recorded possible as non-hemolysis (γ hemolysis), partial hemolysis with greenish surrounding well (α hemolysis) and complete hemolysis with clear zone (β hemolysis) (FDA's BAM, 2001; Sánchez-Ortiz *et al*., 2015).

3.3.5.6 Bile salt tolerance

Bile salt tolerance was determined by visual observation of bacterial growth to culture on agar plates. TSA plates containing different concentrations of bacteriological bile salt (HIMEDIA) at 2, 4, 6, 8, 10, and 12% (*w*/*v*) were prepared. A loop of an overnight bacterial isolate was used to spread on duplicate plates of each concentration and duplicate plates without bile salt as the control. Plates were incubated at 32ºC for 96 hours and then observed bacterial growth as visible growth or no-growth.

3.3.5.7 Acid tolerance

Acid tolerance was recorded by visual observation of bacterial culture on agar plates after incubating bacterial isolates in PBS adjusting the pH at 2 and 4 for 24 hours. In brief, an overnight loop of bacterial isolate was transferred in 1 ml of PBS solutions at pH 2 or 4. These tubes were incubated overnight at room temperature (25ºC) and a volume of 100 µL of these samples was used to spread on TSA plates. These plates were incubated at 32ºC for 96 hours to observe bacterial growth as visible growth or no-growth.

3.3.5.8 Specific growth rate assay

Specific growth rate (μ) of isolates was determined according to Lindqvist and Barmark (2014). In brief, a volume of 100 µl (9×10^8 cells.ml^{-1}) of approximated cell density) of fresh bacterial preparation was used to mix with 900 µl of PBS (pH7.5) in the duplicates. These samples were incubated at 15, 32, and 42ºC for 24 hours, which represented optimize and extreme conditions as low and high for tilapia culture in Thailand. The optical density (OD_{600}) at the beginning, 8 and 24 hours was recorded.

3.3.5.9 The protocol to select probiotic candidates

Multi-parameter studies were categorized into three groups consisting of general parameters, safety parameters and survival parameters. General parameters included pathogenic antagonism and adhesion assays, which had several articles distributing these properties to select probiotics (Gullian *et al*., 2004; Hjelm *et al*., 2004; Aly *et al*., 2008; El-Rhman *et al*., 2009; Das *et al*., 3013; Del'Duca *et al*., 2013; Abdulla *et al*., 2014; Geraylou *et al*., 2014; Widanarni *et al*., 2015; Etyemez and Balcazar, 2016). The potential of probiotics without antibiotic resistance is a strong recommendation (FAO/WTO, 2006; WTO, 2014) referring to microbial pathogens to contain resistance genes may transfer these genes to human pathogens whose cannot treat disease infection using antibiotics. According to hemolytic activity is very important of probiotic properties, which display non-hemolytic to the blood host. Then, both antibiotic resistance and hemolytic parameters are indicated the safety use. Growth and survival parameters as probiotic qualities to survive in the GI environment have been suggested to select probiotics (Vine *et al*., 2004; Mourad and Nour-Eddine, 2006; Balcázar *et al*., 2008; Geraylou *et al*., 2014). Therefore, parameters and sub-parameters were determined to get different scores. Finally, the coefficient index was then calculated by using these scores (Table 3.1). The score of isolates was calculated by using results *in vitro* assays, which had assumptions following:

(I) A total score of 100 was given isolates inhibiting two pathogens and 50 for inhibiting only one pathogen.

(II) A score of 100 was given isolates displaying the highest average percentages of adhesion/ auto-aggregation/ hydrophobicity/ specific growth rate, and then the rest scores were calculated the norm with the highest value.

(III) A score of −100 was allocated to isolates showing the highest numbers of R to antibiotics tested (12 antibiotic discs), −50 for I and 100 for S and then another rest was scored by normalizing

with the highest value; finally, each isolate was allocated a score by summarizing these scores of R, I and S.

(IV) A score of −100 was given to isolates displaying blood hemolysis and 100 without hemolysis.

(V) A total score of 100 was given to isolates tolerating to bile salts at 12% and 50 for tolerating to bile salts at 6%.

(VI) A score of 100 was given to isolates tolerating to pH 2 and 0 displaying non-tolerance.

The score of bacterial isolates was calculated using the following equations:

$$T_i = ci_1 S_{1i} + ci_2 S_{2i} + ci_3 S_{3i} + \cdots ci_n S_{ni}$$

Where: T is the total score of each isolate, ci is the coefficient index, and S is the isolated score of each parameter *in vitro* trials.

The Z−score was calculated by using the following equation:

$$Z_i = \frac{\sum (T_i - \bar{T})}{\sqrt{\frac{\sum_1^n (T_i - \bar{T})^2}{n-1}}}$$

Where: T_i is the total score of isolated bacterial i, $\bar{T}$ is the overall mean score, and n is the total isolate number.

Table 3.1 Summary of determination scores to calculate the coefficient index

Parameter (*P*)	**Parameter Score (*PS*)**	**Sub-parameter (*Sp*)**	**Sub-parameter score (*SpS*)**	**Estimated Score (*ES*):** ***ES=SpS×PS/100)***	**Coefficient index (*ci*):** ***ci=ES×100***
General parameters	30	Pathogenic resistance	10	3	0.03
		Adhesion to the tilapia intestinal cells	50	15	0.15
		Adhesion to hydrocarbon solvents	20	6	0.06
		Auto-aggregation	20	6	0.06
Safety parameters	50	Antibiotic susceptibility test	50	25	0.25
		Hemolytic testing	50	25	0.25
Survival parameters	20	Bile salt tolerance	20	4	0.04
		Acid tolerance	50	10	0.10
		Temperature exposure	30	6	0.06
Total	100			100	1.00

3.3.6 Data analysis

Percentages of adhesion to the intestinal epithelial cells, hydrocarbon solvents, auto-aggregations, and temperature exposures were calculated by using the following equation:

$$\% \text{ Parameter} = \left(\frac{A_t - A_o}{A_o}\right) x100$$

Where, A_t represents the absorbance at time *t* and A_o is the absorbance at the initial absorbance.

Specific growth rate (μ) of isolates was measured by using the following equation:

$$\mu = \left(\frac{\ln OD_n - \ln OD_0}{t_n - t_0}\right)$$

Where, OD_n represents the absorbance at time t_n and $OD_0$is the absorbance at the initial absorbance.

All percentages of parameter studies are transformed to normal distribution. The calculation of these data was performed to check for significant differences within isolates by using one-way analysis of variance (ANOVA). Statistical significance was accepted at $P \leq 0.05$, which was then followed with pairwise comparison probabilities for comparing different isolates. These data were analyzed by using the Systat software ver. 5.02 (Illinois, USA).

3.4 Results

3.4.1 The total colony counts (TCC) and microbial isolation

The TCC (cfu.g^{-1}) in the GI tract of tilapia culturing in MRS-agar, TSA, and NA plates were in ranges of $1.0–4.0\times10^{2}$, $5.4\times10^{6}–2.7\times10^{7}$, and $3.2\times10^{8}–1.3\times10^{9}$, respectively. Microbial loads in the same medium were found non-significant difference ($P>0.05$) between different groups of tilapia (Table 3.2).

Table 3.2 Bacterial loads (mean ± standard deviation: (n) in the tilapia intestine from different sources based on colony forming unit (cfu.ml^{-1}).

Sources of tilapia (N)	MRS-agar	TSA	NA
Group 1: (5)	$4.7\times10^{3}\pm2.8\times10^{3}$	$1.28\times10^{7}\pm5.2\times10^{6}$	$6.2\times10^{8}\pm3.0\times10^{8}$
Group 2: (4)	$4.8\times10^{2}\pm1.5\times10^{2}$	$2.6\times10^{7}\pm3.5\times10^{7}$	$1.2\times10^{9}\pm1.3\times10^{9}$
Group 3: (4)	$2.0\times10^{3}\pm1.1\times10^{3}$	$8.9\times10^{6}\pm5.8\times10^{6}$	$8.5\times10^{8}\pm1.6\times10^{8}$
Group 4: (4)	$4.0\times10^{3}\pm2.6\times10^{3}$	$1.17\times10^{7}\pm2.3\times10^{6}$	$9.4\times10^{8}\pm3.1\times10^{8}$
Group 5: (2)	$6.4\times10^{2}\pm1.1\times10^{2}$	$8.6\times10^{6}\pm2.5\times10^{6}$	$5.7\times10^{8}\pm1.3\times10^{8}$

A total of 265 microbial colonies (41 from MRS-agar, 124 from TSA and 100 from NA plates) were isolated. These colonies were sub-cultured, streaked, and re-streaked on TSA plates for purification. Only bacterial isolates were classified into simple groups by using morphological colony and Gram-stain characterizations. Finally, we found thirty-four isolates having different characterizations, and these isolates displayed to be colonial consistency.

3.4.2 Antagonistic screening

Eight of fifteen isolates were able to inhibit both bacterial pathogens *A. hydrophila* and *S. iniae*. Fourteen isolates were able to inhibit *A. hydrophila,* and nine isolates were against *S. iniae* (Table 3.3). Finally, fifteen isolates with inhibitory activities were accepted as potential probiotic candidates, and subjected to furthure testing.

Table 3.3 *In vitro* tests of the intestinal bacterial isolates showed inhibition against pathogenic bacteria *A. hydrophila* and *S. iniae*.

Isolate no.	Pathogenic bacteria	
	A. hydrophila	***S. iniae***
1	−	+
2	+	+
3	+	+
4	+	+
5	+	−
6	+	+
7	+	+
8	+	+
9	+	−
10	+	−
11	+	−
12	+	+
13	+	−
14	+	−
15	+	−

+ = inhibition , **–** = non-inhibition

3.4.3 Phenotypic characterizations of probiotic bacterial candidates

Most of bacterial isolates had a circular whole colony, entive colony edge, convex elevation, opaque colony color, and 0.1−0.5 mm of diameter. Bacterial isolate no.14 displayed different morphology (filamentous and lobated colony), no.15 and 23 had a flat colony, and isolates no.18, 21, and 23 had a diameter less than 1 mm. Three isolates (13 to 15) were Gram-positive cocci-shaped bacteria, eleven isolates (1 to 10) were Gram-positive rod-shaped bacteria, and three isolates (11 to 12) were Gram-negative with rod-shaped bacteria. All Gram-positive with rod-shaped bacteria displayed endospores in the cells.

The morphological and biochemical characters as carbohydrate fermentation test (glucose, lactose, sucrose, maltose, and mannitol), triple sugar iron (TSI), methyl red, Voges–Proskauer, citrate utilization, oxidation-fermentation test (O-F test), oxidase, catalase, dihydrolase test (lysine, ornithine and arginine), indole production, motility, granule, endospore and capsule were presented in Table 3.4.

Table 3.4 Bacterial characterizations and biochemical tests of bacterial colonies isolated from the intestine of tilapia.

Bacterial isolates	Shape (Gram stain)	Biochemical tests																		The other characteri-zations	
		Glucose Ferm.	Lactose Ferm.	Mannitol Ferm.	Sucrose Ferm.	Moltose Ferm.	TSI-(slant/butt; Gas)	Mehtyl red	Voges-Proskuer	Citrate utilisation	O-F-Faraffin	O-F-Non-faraffin	Oxidase	Catalase	Lysine	Ornithine	Arginine	Indole	Motility	Granule	Endospore	Capsule
1	Rod (+)	−	−	−	−	−	A/A; −	−	−	−	−	−	V	+	−	−	−	−	−	+	+	+
2	Rod (+)	−	−	−	−	−	A/A; +	+	+	−	−	−	V	+	−	−	+	−	+	+	+	+
3	Rod (+)	−	−	−	−	−	A/A; +	+	+	+	V	V	V	+	−	−	+	−	+	+	+	+
4	Rod (+)	−	−	−	−	−	K/A; +	+	+	−	V	V	V	++	−	−	+	−	+	+	+	+
5	Rod (+)	−	−	−	−	−	A/A; −	−	−	+	V	V	−	+	V	V	V	−	−	+	+	+
6	Rod (+)	−	−	−	−	−	K/A; +	+	+	+	V	V	−	++	−	−	+	−	+	+	+	+
7	Rod (+)	−	−	−	−	−	A/K; −	−	−	+	V	V	−	++	V	V	−	−	−	+	+	+
8	Rod (+)	−	−	−	−	−	A/K; −	−	−	+	V	V	−	++	V	V	−	−	−	+	+	+
9	Rod (+)	−	−	−	−	−	A/K; −	−	−	+	V	V	−	+++	V	V	V	−	−	+	+	+
10	Rod (+)	−	−	−	−	−	K/A; +	+	−	−	V	V	V	+++	−	−	+	−	−	+	+	+
11	Rod (−)	+	+	+	+	+	K/K; +	+	−	−	+	+	+	−	+	+	+	+	+	−	−	+
12	Rod (−)	+	+	+	+	+	K/A; +	−	+	+	+	+	−	−	V	V	+	−	+	−	−	+
13	Coccus (+)	−	−	−	−	−	A/K; −	+	+	−	V	V	V	+++	−	−	−	−	−	−	−	+
14	Coccus (+)	−	−	V	V	−	A/K; −	−	−	−	V	V	−	−	−	−	−	−	−	−	−	+
15	Coccus (+)	V	−	−	−	V	A/K; −	−	−	−	V	V	V	+	−	−	−	−	−	−	−	+

+ = Positive; ++=rather strong positive; +++ = the most strong positive; − = Negative; V= variable (mostly positive with some negative); A (slant)=ferments lactose and/or sucrose (yellow); K (slant)=does not ferment either lactose or sucrose (red); A (butt)= some fermentation has occurred, acid has been produced, it is a facultative anaerobe (yellow); K (butt)= no fermentation, the bacterium is an obligate aerobe (red).

3.4.4 16S rDNA identification

The PCR amplification was expected size (1500 bp) of a fragment from the 16S rRNA gene for the fifteen isolates for indicating bacterial identification. BLAST searches results using the obtained sequences revealed the closest know neighbors (see Table 3.5). Ten isolates were identified as *Bacillus* spp. (isolates: RP01, CHP00, NP00, NP01, RP00, CHP01, CHP02, RC00, RC01 and RC02), two as *Staphylococcus* spp. (isolates: CHP04 and NP04), two as *Enterbactor* spp. (isolates: NP03 and NP02) and one as *Macrococcus caseolyticus* (isolate CHP03).

Table 3.5 Summary of the intestinal bacterial identification by using 16S rDNA.

List	Related species (BLAST searching)	Similarity (%)	Reference in GenBank	Strain of this study
1	*B. megaterium*	94	HM480340.1	RP01
	B. aryabhattai	98	JQ905075.1	
2	*B. cereus*	98	DQ339648.1	CHP00
3	*B. cereus*	98	KF032688.1	NP00
4	*B. cereus*	96	KJ948667.1	NP01
5	*Bacillus* sp.	97	KC429572.1	RP00
6	*Bacillus* sp.	95	JX307075.1	CHP01
	B. cereus	95	KF032688.1	
7	*Bacillus* sp.	93	JF701958.1	CHP02
8	*B. megaterium*	99	KJ767327.1	RC00
	B. aryabhattai	99	KF933685.1	
9	*B. megaterium*	88	KJ009493.1	RC01
	B. aryabhattai	88	JQ236819.1	
10	Uncultured *Bacillus* sp.	94	KP016675.1	RC02
	Bacillus sp.	94	HE662657.1	
11	*Ent. asburiae*	97	HQ407265.1	NP03
	Enterobacter sp.	97	KF896099.1	
12	*Ent. sakazakii*	86	KF360280.1	NP02
	Cro. sakazakii	86	FJ906914.1	
13	*Mac. caseolyticus*	97	KJ638988.1	CHP03
14	*Stap. arlettae*	97	KP753921.1	CHP04
15	*Stap. sciuri*	97	HQ154558.1	NP04

3.4.5 *In vitro* trials

3.4.5.1 Adherence assay to tilapia intestinal cells

The adhesive levels of probiotic candidates were no significantly different (P>0.05) for the two incubation periods of 2 and 6 hours, except the adhesive-potential incubated for 4 hours (P<0.5) (Table A.2 of Appendix 2). The adhesion abilities of isolates were tended to increase with exposure times of 2, 4 and 6 hours (Figure 3.1 & 3.2), which were 4.67±1.36, 7.52±1.19, and 10.10±2.64%, respectively. High adhesive potential of exposure times were found for three *Bacillus* strains as *Bacillus* sp. CHP02, *B. cereus* NP01 and *Bacillus* sp. RP01, which had 13.05±1.67, 11.29±1.15, and 10.70±2.75%, respectively. Conversely, low adhesive potentials were displayed by *Enterobacter* sp. NP03, *Stap. sciuri* NP04, and *Bacillus* sp. RC02 which had 4.34±2.67, 2.78±1.92, and 2.71±2.99%, respectively. The adhesive potential of the pathogenic *A. hydrophila* and *S. iniae* strains used in this study were 3.62±0.73, and 1.35±1.06%, respectively.

3.4.5.2 Adhesion to hydrocarbon solvents

The abilities of adhesive-potential of isolates to chloroform and hexane (Table A.3 & A.4 of Appendix 2) studied. A greater adhesion to chloroform than hexane was observed, 45.37±2.89 and 8.55±0.92%, respectively (Figure 3.3). Bacterial isolates of *Enterobacter* sp. NP02 (94.10±0.48%), *Stap. sciuri* NP04 (80.84±3.37%) and *Bacillus* sp. CHP02 (74.49±3.09%) displayed the highest adhesions to chloroform, and the lowest adhesions were found for *Enterobacter* sp. NP03 (24.45±2.98%), *Stap. arlettae* CHP04 (14.71±0.07%) and *Bacillus* sp. RC02 (9.11±6.31%). Moreover, adhesion to chloroform for *A. hydrophila* and *S. iniae* was 71.58±5.74, and 42.58±3.71%, respectively.

The highest adhesions to hexane were occurred with *Enterobacter* sp. NP02 (48.58±0.38%), *Bacillus* sp. RC02 (18.08±1.06%) and *Stap. sciuri* NP04 (14.12±1.36%). On the other hand, the lowest adhesions were observed for *B. cereus* NP01 (3.52±1.66%), *Bacillus* sp. CHP01

(3.45±0.51%) and *Stap. arlettae* CHP04 (0.52±0.34%). High adhesive capacity to both hydrocarbons was displayed by *Enterobacter* sp. NP02 and *Stap. sciuri* NP04. Despite *Bacillus* sp. RC02 showing a high potential of adhesion to chloroform it displayed a low adhesive capacity to hexane. Finally, the ability of adhesions to hexane for *A. hydrophila* and *S. iniae* was to be 20.91±1.09, and 6.48±0.24%, respectively.

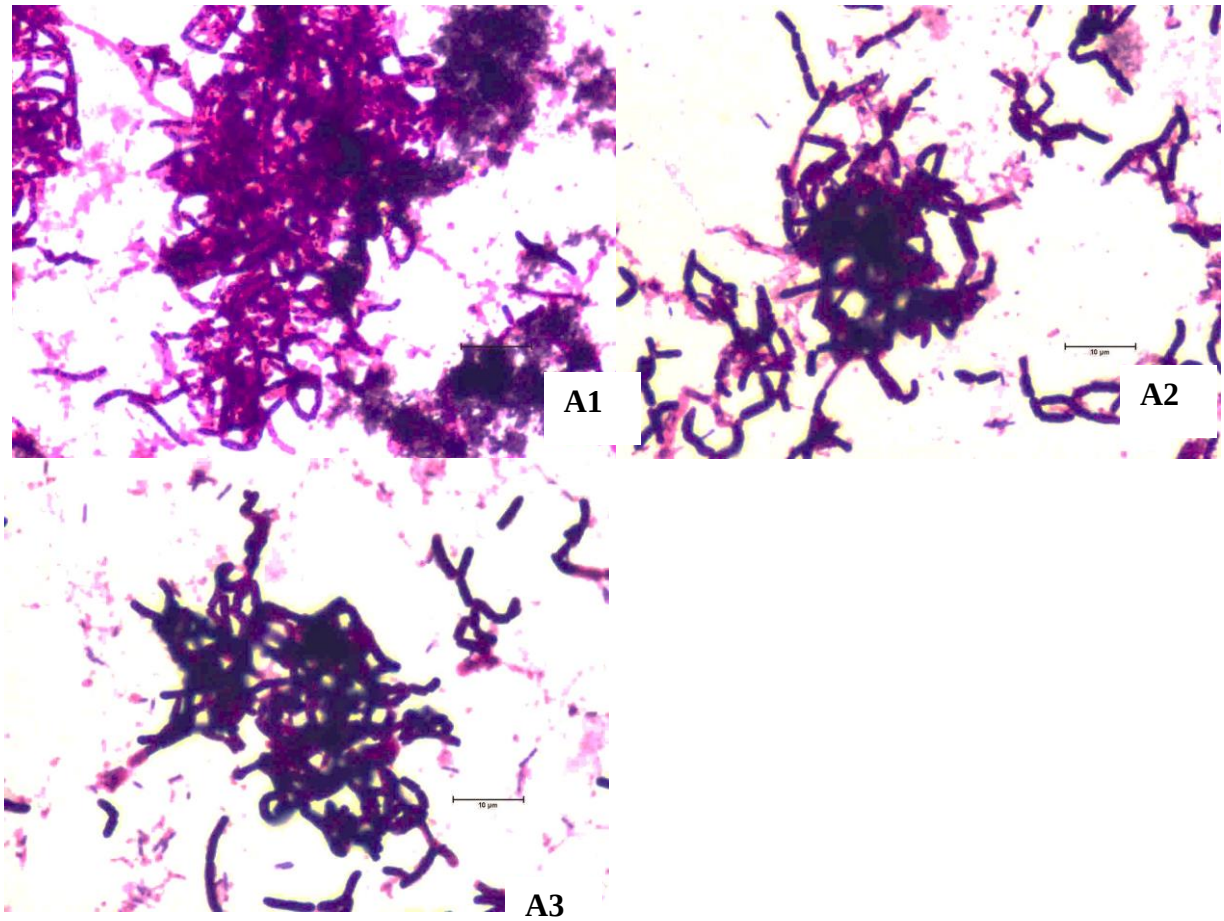


Figure 3.1 Adhesion of *Bacillus sp.* RP00; A1: adhesion at 2 hours, A2 adhesion at 4 hours, and A3: adhesion at 6 hours (scale bar=10 μm).

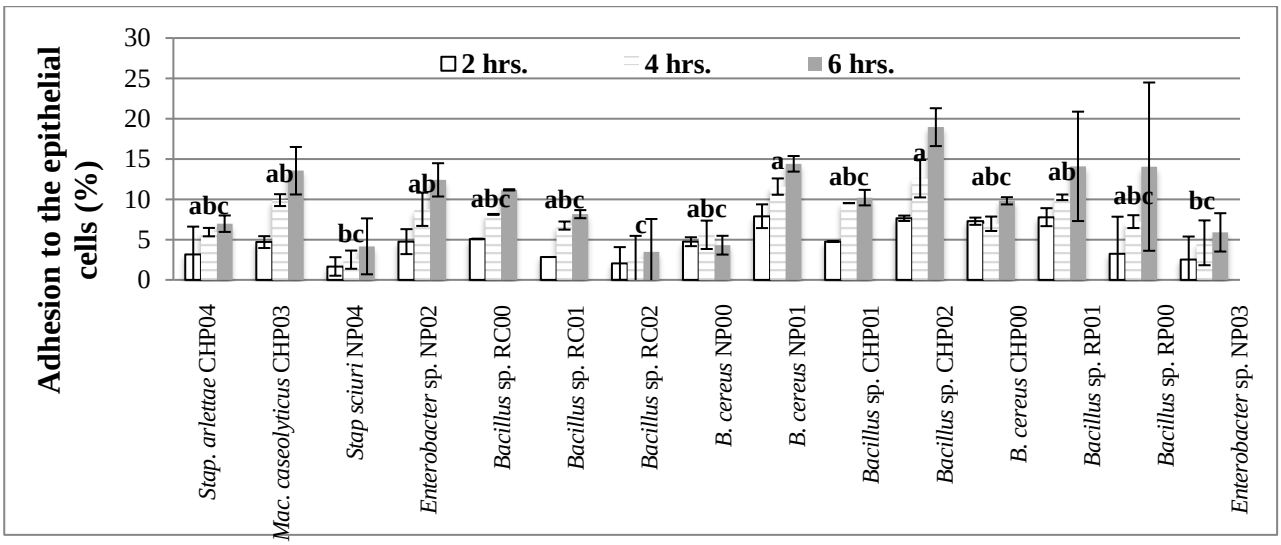


Figure 3.2 Adhesive percentages to the tilapia epithelial cells at different time exposures of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences (P<0.05) in each time.

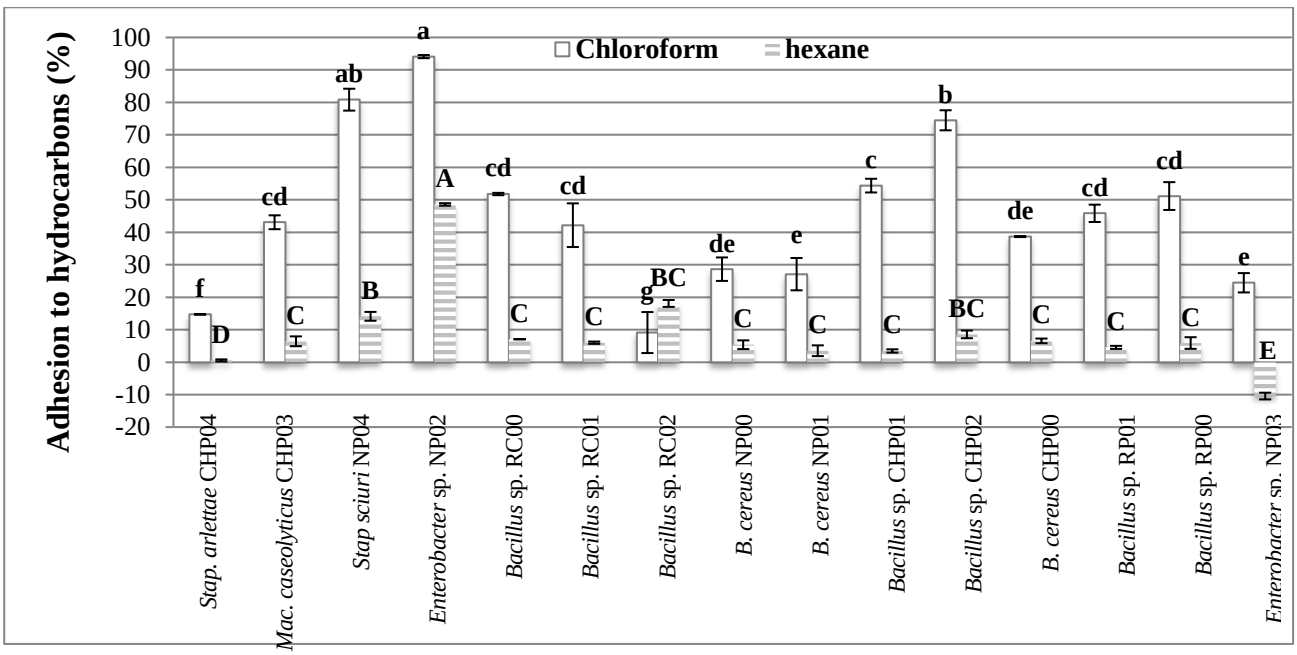


Figure 3.3 The adhesive abilities to hydrarbons of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences (P<0.05) in each time.

3.4.5.3 Auto-aggregation assays

There were significant differences (P≤0.05) between auto-aggregation in PBS of isolates at 4 and 6 hours of incubation times (Figure 3.4 & Table A.5 & A.6 in Appendix 2) and non-difference (P>0.05) was observed at 2 hours. The highest abilities of three exposure times in PBS were observed for *Stap. sciuri* NP04 (48.38±7.79%), *Bacillus* sp. RC02 (41.41±0.92%) and *Mac. caseolyticus* CHP03 (35.86±1.54%) and the lowest adhesive-potentials were displayed as *Stap. arlettae* CHP04 (28.40±1.02%), *Bacillus* sp. RP01 (27.92±3.93%), and *B. cereus* NP00 (23.59±1.03%). Auto-aggregations in PBS of *A. hydrophila* and *S. iniae* was 36.52±1.22, and 24.24±4.87%, respectively.

Statistically significant differences (P≤0.05) of auto-aggregation in sterile 0.85% NaCl during three times of incubations were observed (Figure 3.5 & Table A.7, A.8 & A.9 in Appendix 2). The highest abilities were detected in *Bacillus* sp. RC02 (43.09±2.24%), *Stap. sciuri* NP04 (33.13±4.74%), and *Mac. caseolyticus* CHP03 (27.27±0.47%). The lowest adhesive abilities in sterile 0.85% NaCl were displayed by *Enterobacter* sp. NP02 (16.31±0.74%), *B. cereus* CHP00 (16.02±5.53%) and *Bacillus* sp. RP01 (15.79±1.98%). In addition, bacterial pathogens: *A. hydrophila* and *S. iniae* were to be 27.72±2.22, and 10.25±0.47%, respectively.

The increasing of auto-aggregations in both buffer solvents tended to depend on incubation times. Bacterial isolates displayed high adhesions in PBS than in sterile 0.85% NaCl. High adhesions in both PBS and sterile 0.85% NaCl were observed for *Stap. sciuri* NP04, *Bacillus* sp. RC02 and *Mac. caseolyticus* CHP03, while low adhesive isolates in both buffers were displayed by *B. cereus* CHP00 and *Bacillus* sp. RP01.

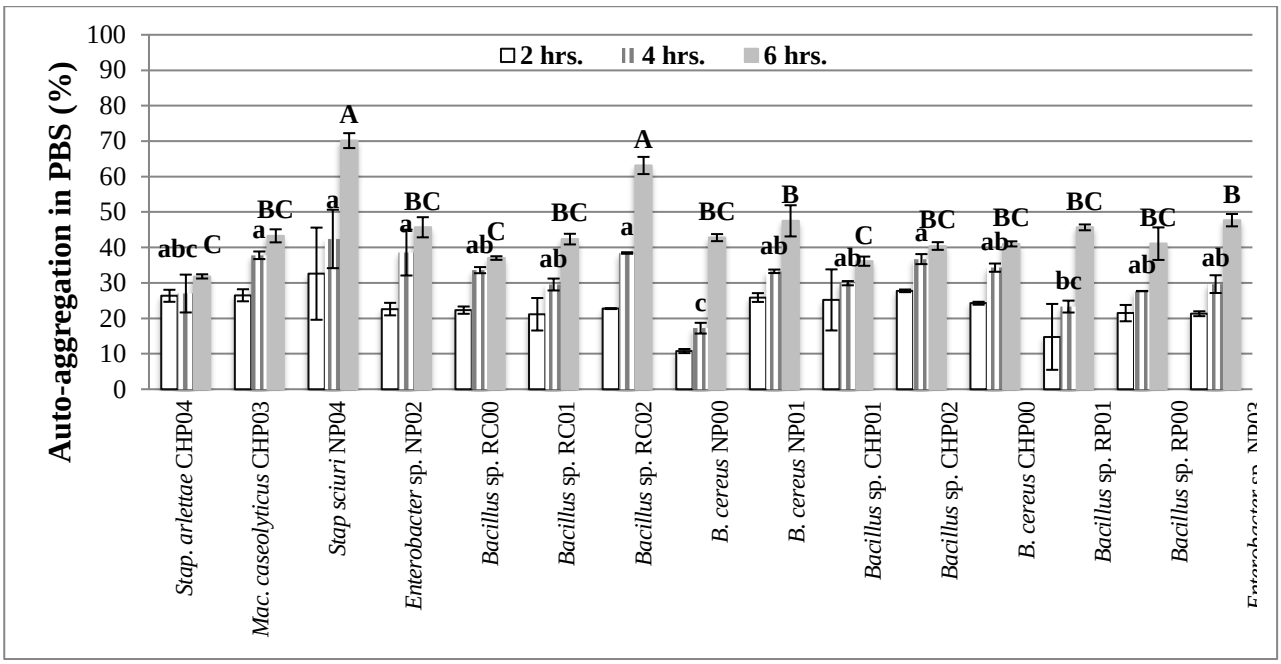


Figure 3.4 Auto-aggregation percentages at different time exposures in PBS of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences ($P<0.05$) in each time.

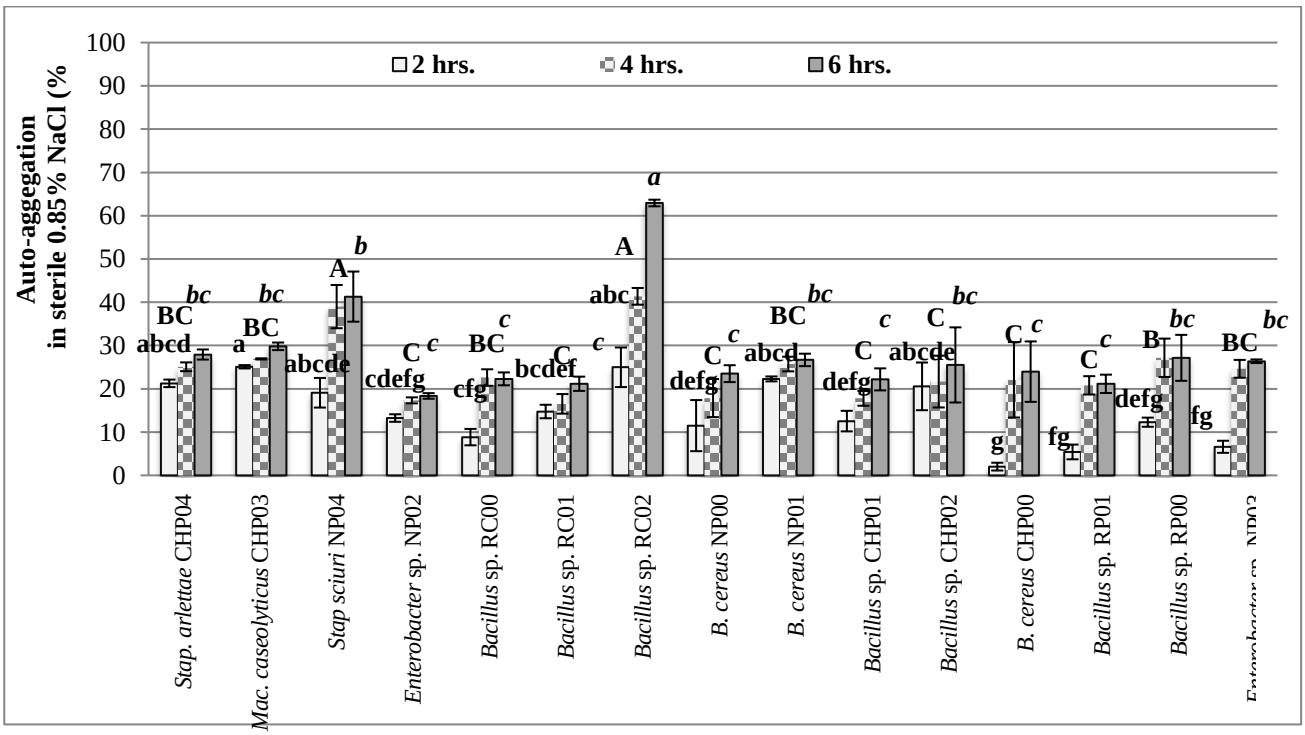


Figure 3.5 Auto-aggregation percentages at different time exposures in sterile 0.85% NaCl of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences ($P<0.05$) in each time.

3.4.5.4 Antibiotic susceptibility test

Fifteen isolates showed some degree of resistance to the antibiotics tested (Table 3.6). Seven isolates: *Bacillus* sp. RP01, *Bacillus* sp. RP00, *Bacillus* sp. CHP02, *Bacillus* sp. RC00, *Bacillus* sp. RC01, *Mac. caseolyticus* CHP03 and *Stap. sciuri* NP04 displayed sensitivity to all antibiotic discs. Eight of the fifteen isolates were resistant to at least one of the antibiotics. Three isolates of *Bacillus cereus* (CHP00 NP00 and NP01) and *Bacillus* sp. RC02 were resistant to Sulphamethoxazole/Thrimethoprim, and two strains of *Enterobactor* sp. (NP02 and NP03) and *Stap. arlettae* CHP04 were resistant to erythromycin. Only *Bacillus* sp. CHP01 showed multi-resistance to ampicillin, cephalothin and sulphamethoxazole/thrimethoprim. Moreover, two isolates of *Bacillus* sp. RC00 and *Enterobactor* sp. NP02 displayed intermediate resistance to ampicillin and neomycin, respectively.

3.4.5.5 Hemolytic activities

All *B. cereus* strains (CHP00, NP00, and NP01) and *Bacillus* spp. (CHP01 and RC02) displayed consistent β-hemolysis for sheep blood and tilapia blood (Table 3.7). Ten isolates were non-hemolytic for both blood types. *B. cereus* isolates CHP00, NP00 and NP01 showed greater hemolytic activities to tilapia blood than sheep blood, with clearing zones measuring 25−26 mm for tilapia blood and 19−22 mm for sheep blood. *Bacillus* sp. CHP01 displayed equal hemolytic activities to both blood agars (6-9 mm). However, *Bacillus* sp. RC02 displayed greater haemolysis of tilapia blood (18−19 mm) than sheep blood (9−11 mm). The pathogenic *A. hydrophila strain* affected both blood types activities, with 6 mm clearance of sheep blood and 16−18 mm of tilapia blood. *S. iniae* displayed equal hemolysis of both blood types (6−8.5 mm).

Table 3.6 Antibiotic susceptibility to 12 antibiotics tested of potential probiotics.

Antibiotic disc	Bacterial isolates														
	Bacillus sp. RP01	*B. cereus* CHP00	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Enterobacter* sp. NP03	*Enterobactor* sp. NP02	*Mac. caseolyticus* CHP03	*Stap.arlettae* CHP04	*Stap. sciuri* NP04
Ampicillin 10 μg: AMP10	S	S	S	S	S	R	S	I	S	S	S	S	S	S	S
Cephalothin 30 μg: KF30	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S
Gentamycin 10 μg: CN 10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin 30 μg: K 30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin 30 μg: N 30	S	S	S	S	S	S	S	S	S	S	I	S	S	S	S
Enrofloxacin 5 μg: ENR 5	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin 15 μg: E 15	S	S	S	S	S	S	S	S	S	S	R	R	R	S	S
Tetracycline 30 μg: TE 30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Oxolinic acid 2 μg: QA 2	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Oxytetracycline 30 μg: OT 30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Nitrofurantoin 300 μg: F 300	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole/Thrimethoprim 25 μg: SXT 25	S	R	R	R	S	R	S	S	S	R	S	S	S	S	S

S=susceptible; I=intermediate and R=resistant

Table 3.7 Hemolytic activities of probiotic candidates on sheep blood and tilapia blood.

Bacterial isolates	Hemolytic activities (mm)	
	Sheep blood	**Tilapia blood**
Bacillus sp. RP01	γ hemolysis	γ hemolysis
B. cereus CHP00	β hemolysis (19.5±0.71)	β hemolysis (26.0±0.00)
B. cereus NP00	β hemolysis (19.5±0.71)	β hemolysis (25.0±0.00)
B. cereus NP01	β hemolysis (22±1.41)	β hemolysis (26±0.00)
Bacillus sp. RP00	γ hemolysis	γ hemolysis
Bacillus sp. CHP01	β hemolysis (9.0±0.00)	β hemolysis (7±1.41)
Bacillus sp. CHP02	γ hemolysis	γ hemolysis
Bacillus sp. RC00	γ hemolysis	γ hemolysis
Bacillus sp. RC01	γ hemolysis	γ hemolysis
Bacillus sp. RC02	β hemolysis (10.5±0.71)	β hemolysis (19.5±0.71)
Enterobacter sp. NP03	γ hemolysis	γ hemolysis
Enterobacter sp. NP02	γ hemolysis	γ hemolysis
Mac. caseolyticus CHP03	γ hemolysis	γ hemolysis
Stap. arlettae CHP04	γ hemolysis	γ hemolysis
Stap. sciuri NP04	γ hemolysis	γ hemolysis

3.4.5.6 Bile salt tolerance

All bacteria tested were able to tolerate the minimum concentration at 6%. However, two strains of *Bacillus* spp. (RP01 & RC00), two strains of *Enterobacter* spp.(NP02 & NP03), and two strains of *Staphylococcus* spp. (CHP04 & NP04) tolerated 8% of bile salt concentrations. The highest tolerance at 12% of bile salt concentrations was found in *Bacillus* sp. RP01, *Enterobacter* sp. NP03, *Stap. arlettae* CHP04 and *Stap. sciuri* NP04 (Table 3.8).

3.4.5.7 Acid tolerance

The ability of isolates to resist the low acidic conditions was found that isolates performing under pH 4 for 24 hours, which displayed growth on agar plates. The highest resistance, to pH 2 for 24 hours, was observed in all *Bacillus* strains, while the other isolates of *Enterobacter* sp. NP03 and NP02, *Mac. caseolyticus* CHP03, *Stap. arlettae* CHP04 and *Stap. sciuri* NP04 were unable tolerate to pH 2 (Table 3.8).

Table 3.8 Assessment growth of bacterial isolate after stimulating at different levels of bile salts and pH

Bacterial isolates	**% Bile salts**			**pH**	
	8	**10**	**12**	**2**	**4**
Stap. arlettae CHP04	1	1	1	0	1
Mac. caseolyticus CHP03	1	1	0	0	1
Stap. sciuri NP04	1	1	1	0	1
Enterobacter sp. NP02	1	1	1	0	1
Bacillus sp. RC00	1	1	1	1	1
Bacillus sp. RC01	0	0	0	1	1
Bacillus sp. RC02	0	0	0	1	1
Ba. cereus NP00	0	0	0	1	1
Bacillus sp. NP01	0	0	0	1	1
Bacillus sp. CHP01	0	0	0	1	1
Bacillus sp. CHP02	0	0	0	1	1
Ba. cereus CHP00	0	0	0	1	1
Bacillus sp. RP01	1	1	1	1	1
Ba. megaterium RP00	0	0	0	1	1
Enterobacter sp. NP03	1	1	1	0	1

0 = non-visible growth; 1=visible growth

3.4.5.8 Specific growth rate

Bacterial isolates were treated at different temperatures (15, 32 and 42^{o}C) to monitor bacterial changes by using the parameter of the specific growth rates within 8 and 24 hours. Significant differences ($P \leq 0.05$) of specific growth rate at three different temperatures both within 8 and 24 hours were found (Table A.10, A.11, A.12, A.13, A.14 & A.15 in Appendix 2). Overall isolates displayed to increase changes in different temperatures of 15, 32 and 42^{o}C (0.061±0.018, 0.172±0.113 and 0.185±0.134, respectively). Although a greater average of bacterial changes were founded in 8 (0.202±0.112) than 24 hours (0.077±0.025). However, highest increasing was displayed to be in 8 hours of 42 and 32^{o}C, while the lowest was found in low temperature at 15^{o}C.

At 15ºC of the incubation times after 8 and 24 hours (Figure 3.6), the highest specific growth rates were found in *Stap. sciuri* NP04 (0.139±0.002), *Bacillus* sp. RP01 (0.139±0.007), and *Enterobacter* sp. NP03 (0.138±0.002), while the lowest averages in *B. cereus* CHP00 (0.004±0.003), *B. cereus* NP01 (0.001±0.003) and *Bacillus* sp. RC02 (−0.009±0.001). At 32ºC of incubation times of 8 and 24 hours (Figure 3.7), the highest averages were found in *Bacillus* sp. RP00 (0.212±0.005), *Mac. caseolyticus* CHP03 (0.187±0.001) and *Bacillus* sp. CHP01 (0.186±0.001), while the lowest averages in *Enterobacter* sp. NP03 (0.163±0.001), *Bacillus* sp. RC02 (0.156±0.003), and *Enterobacter* sp. NP02 (0.140±0.002). At 42ºC of incubation times of 8 and 24 hours (Figure 3.8), the highest averages were displayed in *Mac. caseolyticus* CHP03 (0.224±0.000), *Bacillus* sp. RP00 (0.220±0.001) and *Stap. arlettae* CHP04 (0.216±0.001), while the lowest averages for *Enterobacter* sp. NP03 (0.154±0.001), *Stap. sciuri* NP04 (0.154±0.001) and *Enterobacter* sp. NP02 (0.150±0.001).

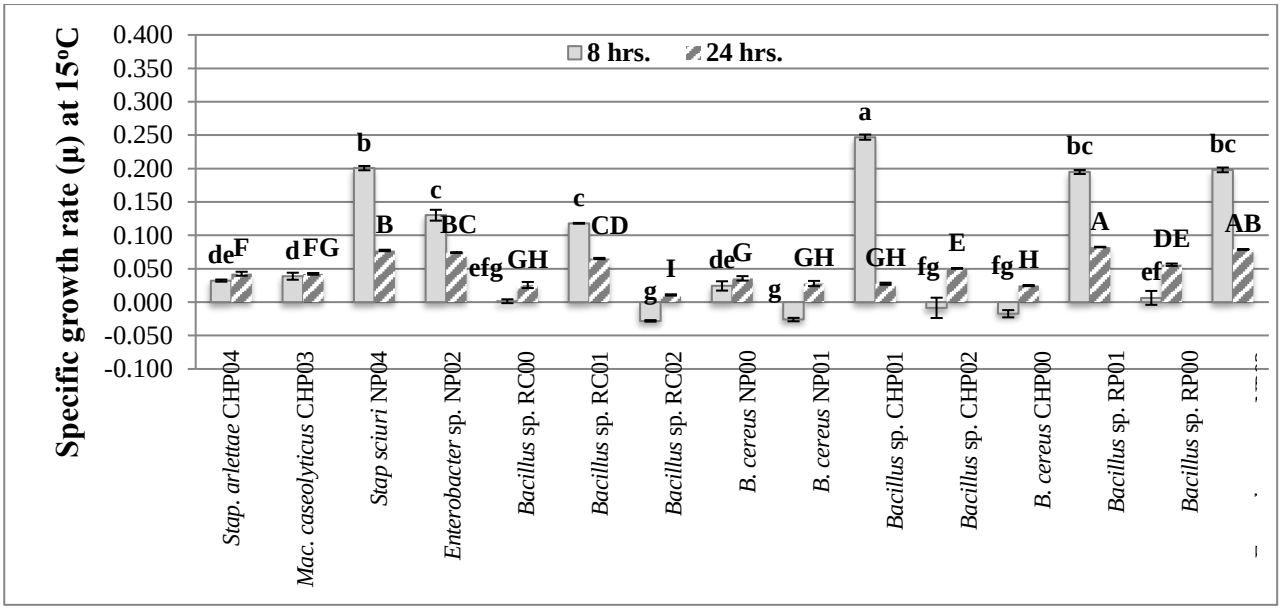


Figure 3.6 Specific growth rates at 15ºC within 8 and 24 hours of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences (P<0.05) in each time.

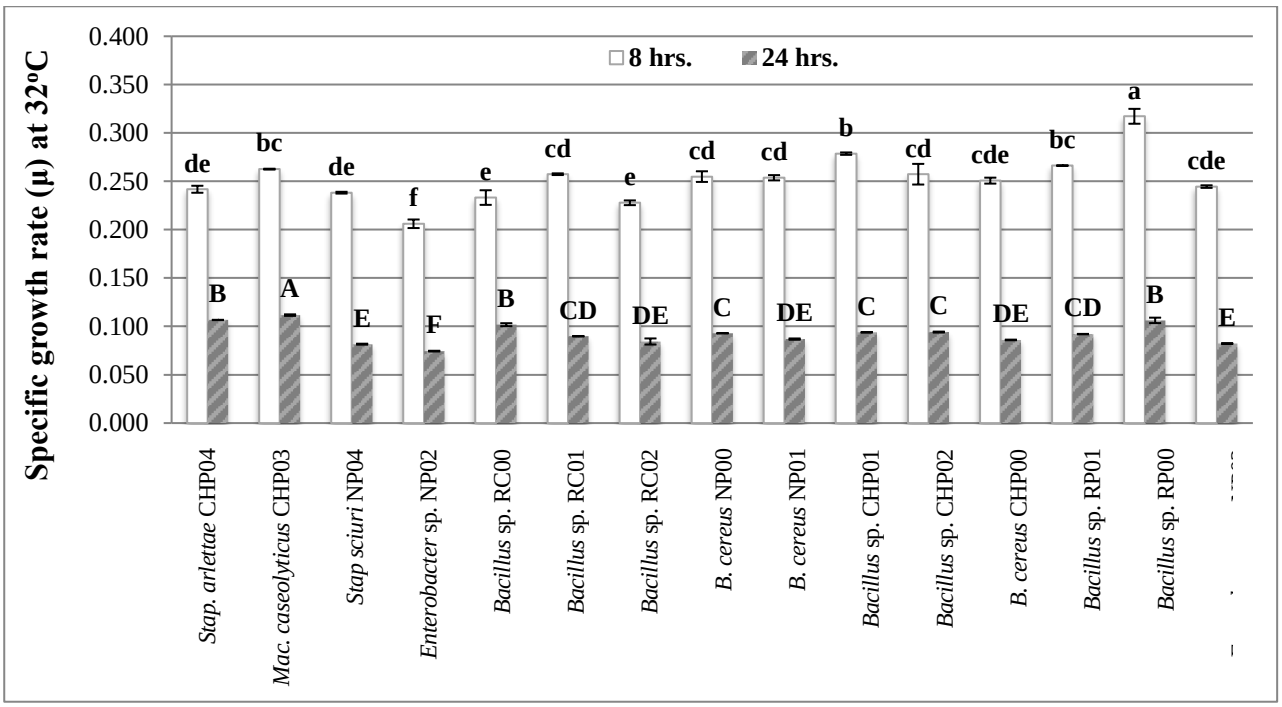


Figure 3.7 Specific growth rates at 32ºC within 8 and 24 hours of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences (P<0.05) in each time.

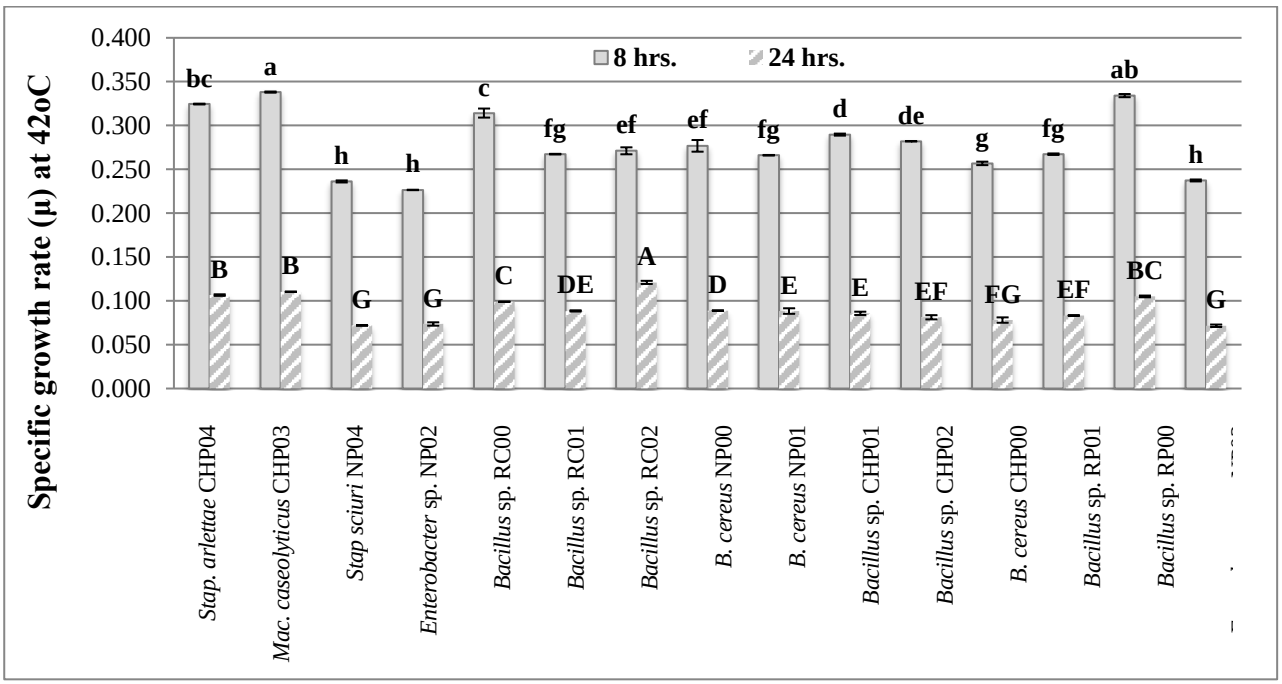


Figure 3.8 Specific growth rates at 42ºC within 8 and 24 hours of potential probiotics. Standard error of the mean bars (n=2) and different letters in column denote significant differences (P<0.05) in each time.

3.4.5.9 Probiotic candidate selection

The results of the multi-parameter data of isolates were converted to numeric scores (Table A.16 & A.17 in Appendix 3), which had totaled 900 from nine parameters. The ranking of total score of fifteen isolates were displayed as *Bacillus* sp. CHP02 (711), *Bacillus* sp. RP01 (705), *Bacillus* sp. RC00 (676), *Enterobacter* sp. NP02 (657), *Bacillus* sp. RP00 (643), *Bacillus* sp. RC01 (613), *Stap. sciuri* NP04 (605), *Stap. arlettae* CHP04 (542), *Mac. caseolyticus* CHP03 (517), *B. cereus* NP01 (447), *Enterobacter* sp. NP03 (431), *B. cereus* CHP00 (416), *Bacillus* sp. CHP01 (388), *B. cereus* NP00 (387) and *Bacillus* sp. RC02 (363). Briefly, the description of Z-score calculation was begun to use results of *in vitro* trials transforming to numeric scores and then multiplied with the coefficient index of each parameter. Overall mean and square of individual value minus with overall mean were estimated. Then, these scores were used calculations using the Z−score equation (more detail of calculations was expressed in Appendix 3).

The ranking of the Z−score are follows: *Bacillus* sp. CHP02 (1.14), *Bacillus* sp. RP01 (1.09), *Bacillus* sp. RP00 (0.94), *Bacillus* sp. RC01 (0.83), *Stap. sciuri* NP04 (0.63), *Bacillus* sp. RC00 (0.61), *Enterobactor* sp. NP02 (0.50), *Stap. arlettae* CHP04 (0.45), *Mac. caseolyticus* CHP03 (0.32), *Enterobacter* sp. NP03 (−0.37), *B. cereus* NP01 (−0.96), *B. cereus* CHP00 (−1.10), *B. cereus* NP00 (−1.23), *Bacillus* sp. RC02 (−1.28), and *Bacillus* sp. CHP01 (−1.57). These autochthonous bacteria show the ranking of Z scores and probiotic properties in Table 3.9.

Table 3.9 Attributes and scores of autochthonous bacteria originated from the intestine of tilapia.

Isolates (Z-scores)	Antagonistic screening	Adhesion to tilapia epithelial cells	Adhesion to chloroform	Adhesion to hexane	Auto-aggregation in PBS	Auto-aggregation in sterile 0.85% NaCl	Antibiotic susceptibility test	Hemolytic activities	Bile salt tolerance	pH tolerance	Specific growth rate
Bacillus sp. CHP02 (Z = 1.14)	Both pathogens	13.05±1.67	74.49±3.09	8.55±1.18	34.96±0.96	22.58±6.72	S=12	Non-hemolysis	6% bile salts	pH 2	0.126±0.005
Bacillus sp. RP01 (Z = 1.09)	*S. iniae*	10.70±2.75	45.84±2.67	4.55±0.47	27.92±3.93	15.79±1.98	S=12	Non-hemolysis	12% bile salts	pH 2	0.164±0.001
Bacillus sp. RP00 (Z = 0.94)	*A. hydrophila*	8.17±5.28	51.16±4.28	5.89±1.79	30.08±2.31	22.20±3.60	S=12	Non-hemolysis	6% bile salts	pH 2	0.154±0.004
Bacillus sp. RC01 (Z = 0.83)	*A. hydrophila*	5.91±0.33	42.18±6.72	5.99±0.35	31.02±2.59	17.48±1.82	S=12	Non-hemolysis	6% bile salts	pH 2	0.148±0.001
Stap. sciuri NP04 (Z = 0.63)	*A. hydrophila*	2.78±1.91	80.84±3.37	14.12±1.36	48.38±7.78	33.13±4.74	S=12	Non-hemolysis	12% bile salts	pH 4	0.151±0.001
Bacillus sp. RC00 (Z = 0.61)	Both pathogens	8.12±0.06	51.78±0.36	7.06±0.07	30.97±0.80	17.91±1.76	S=11 & I=1(AMP10)	Non-hemolysis	6% bile salts	pH 2	0.129±0.004
Enterobactor sp. NP02 (Z = 0.50)	Both pathogens	8.64±1.89	94.10±0.48	48.58±0.38	35.62±3.69	16.31±0.74	S=11 & R=1 (E 15)	Non-hemolysis	6% bile salts	pH 4	0.131±0.003
Stap. arlettae CHP04 (Z = 0.45)	*A. hydrophila*	5.35±1.67	14.71±0.07	0.52±0.34	28.40±2.54	24.76±1.02	S=12	Non-hemolysis	12% bile salts	pH 4	0.142±0.002
Mac. caseolyticus CHP03 (Z = 0.32)	*A. hydrophila*	9.38±1.47	43.09±2.13	6.43±1.51	35.86±1.54	27.27±0.47	S=11 & R=1 (E 15)	Non-hemolysis	6% bile salts	pH 4	0.151±0.001
Enterobacter sp. NP03 (Z = −0.37)	*A. hydrophila*	4.34±2.67	24.45±2.98	−10.44±1.04	32.89±1.64	19.20±1.29	S=11, I=1 (N 30) & R=1 (E 15)	Non-hemolysis	12% bile salts	pH 4	0.152±0.001

Table 3.9 *Continued…*

Isolates	Antagonistic screening	Adhesion to tilapia epithelial cells	Adhesion to chloroform	Adhesion to hexane	Auto-aggregation in PBS	Auto-aggregation in sterile 0.85% NaCl	Antibiotic susceptibility test	Hemolytic activities	Bile salt tolerance	pH tolerance	Specific growth rate
B. cereus NP01 (Z = −0.96)	Both pathogens	11.29±1.15	27.10±4.98	3.52±1.66	35.55±2.04	24.90±1.23	S=11 & R= 1 (SXT 25)	β hemolysis	6% bile salts	pH 2	0.116±0.002
B. cereus CHP00 (Z = −1.10)	Both pathogens	8.02±0.60	38.68±0.16	6.56±0.71	33.19±0.77	16.02±5.53	S=11 & R= 1 (SXT 25)	β hemolysis	6% bile salts	pH 2	0.113±0.002
B. cereus NP00 (Z = −1.23)	Both pathogens	4.88±1.16	28.64±3.62	5.37±1.35	23.59±1.03	17.62±4.09	S=11 & R=1 (SXT 25)	β hemolysis	6% bile salts	pH 2	0.129±0.004
Bacillus sp. RC02 (Z=−1.28)	*A. hydrophila*	2.17±2.99	9.11±6.31	18.08±1.06	41.44±0.09	43.09±2.42	R=2 (N 30& SXT 25)	β hemolysis	6% bile salts	pH 2	0.115±0.000
Bacillus sp. CHP01 (Z=−1.57)	Both pathogens	8.16±0.32	54.37±2.11	3.45±0.51	30.41±3.31	17.51±2.21	R=3 (AMP10, KF30 & SXT 25)	β hemolysis	6% bile salts	pH 2	0.170±0.002

S=susceptible, I=intermediate, R=resistant and 12 antibiotics: AMP10, KF30, CN 10, K 30, N 30, ENR 5, E 15, TE 30, QA 2, OT 30, F 300 & SXT 25

3.5 Discussion

Bacterial loads in the tilapia GIT depended on culture media, organ studies, seasoning, and cultural system (Spanggaard *et al*., 2000; Al-Harbi and Uddin, 2003; Molinari *et al*., 2003; Brunt and Austin, 2005; Pond *et al*., 2006; Balcázar *et al*., 2007; Wu *et al*., 2010). The Nile tilapia gut has reported to be 2×10^4 to 2×10^5 cfu.g^{-1} of microbial culture by using purified agar (Difco) (Molinari *et al*., 2003), hybrid tilapia were found to be 1×10^7 to 8×10^7 cfu.g^{-1} by using specialist Lab M agar (He *et al*., 2013), the same of hybrid tilapia reared in earthen ponds was estimated to be 2×10^6 to 6×10^7 cfu.g^{-1} using TSA (Al-Harbin and Uddin, 2003). In addition, different seasons of tilapia culture showed microbial variations between 7×10^5 to 4×10^9 cfu.g^{-1} by culturing in TSA plates (Al-Harbin and Uddin, 2004). The microbial loads of the tilapia GI in this study were 1.0-3.7$\times10^2$ in MRS-agar, 5.4×10^6 to 2.7×10^7 in TSA, and 3.2×10^8 to 1.32×10^9 in NA. MRS-agar plates displayed yeast, fungi, and small bacterial colonies. Bacterial loads occurred in NA than TSA plates, however, morphological bacterial diversity was observed in TSA than NA plates and MRS-agar plates.

The potential probiotic is to inhibit pathogenic bacteria, which is an important property. In this study, fifteen of thirty-four isolates were identified to be *Bacillus* spp. (ten isolates), a few isolates were *Enterobacter* spp. (two isolates) and *Staphylococcus* spp. (two isolates), and the other species was *Macrococcus caseolyticus*. These bacteria were shown to inhibit bacterial pathogens (*A. hydrophila* and *S. iniae*), which displayed to inhibit only on *A. hydrophila* or *S. iniae* or on both pathogens. According to several pathogenic bacteria as *A. hydrophila*, (Aly *et al*., 2008b; Balcázar *et al*., 2008; Pan *et al*., 2008; El-Rhman *et al*., 2009; Chantharasophon *et*

al., 2011; Del'Duca *et al*., 2013; Das *et al*., 2013; Kumar *et al*., 2013; Tulini *et al*., 2013; Geraylou *et al*., 2014), *Edwerdsiella tarda*, *Enterococcus faecalis*, *Escherichia coli*, *Flavobacterium columnare*, *Listeria monocytogenes*, *Pseudoalteromonas* sp., *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus* spp., *Vibrio* spp. and *Yersinia ruckeri* were used to evaluate the probiotic property for exhibiting pathogens (Hjelm *et al*., 2004; Balcázar *et al*., 2008; Pan *et al.*, 2008; Apún-Molina *et al*., 2009; Chemlal-Kherraz *et al*., 2012; Del'Duca *et al.*, 2013; Kumar *et al*., 2013; Tulini *et al.*, 2013; Gao *et al*., 2013; Das *et al*., 2013; Geraylou *et al*., 2014; Prieto *et al*., 2014). Two species of pathogens have been reported the causes of the mass mortalities of tilapia culture in Thailand (Yuasa *et al*., 2008; Jantrakajorn *et al*., 2014; Chitmanat *et al*., 2016). Then the potential probiotics inhibit pathogens, which used to consider as high potential probiotic.

Generally, *Bacillus* spp., are rod-shape, spore forming, with granule in cell, and facultative anaerobes. Many commercial *Bacillus* probiotics such as *Bacillus cereus*, *Bacillus clausii*, *Bacillus pumilus*, are general trade for human (Duc *et al*., 2004). Several *Bacillus* spp. such as *B. subtilis*, *B. pumilus* and *B. cereus* are often reported to be present in freshwater ecosystem (Mohanty, *et al*., 2011). The GI tract of tilapias has been reported to identify several *Bacillus* strains (Al-Harbi and Uddin, 2004; Chantharasophon *et al*., 2011; He *et al*., 2013; Del'Duca *et al*., 2013). These strains were also found in both organic fertilizers (poultry, pig, blood meal) and fishes fed with organic fertilizers (Ampofo and Clerk, 2010). Moreover, *B. brevis* was isolated from the GI tract of tilapia and proved to be as a potential probiotic *in vitro* trials (Chantharasophon *et al*., 2011). In this study, the GIT of different areas of tilapia culture were

identified ten isolates to be *Bacillus* spp. and these bacteria were evaluated to be a potential bacterial probiotic.

Enterobacter spp. has been reported to occur generally in aquatic environments, aquatic plants, and landscape (Grimont and Grimont, 2006). The character of Family Enterobacteriaceae has a rod-shape, motile, non-spore forming, without granule in cell, and facultative anaerobes. Currently, *C. sakazakii* was reclassified to *Ent. sakazakii* (FAO/WTO, 2008), this bacterium causes infections in childern. The gut of rainbow trout, yellow catfish, and tilapia have been detected several strains of *Enterobacter* spp. (Pond *et al*., 2006; Boari *et al*., 2008; Wu *et al*., 2010). However, this strain can inhibit bacterial pathogen in this study then it could be expected to be a candidate probiotic. Another species of *Staphylococcus* spp. were isolated from the GIT of tilapia in this study. Although, *Staphylococcus* spp. bacteria have been found in the intestinal tract, gills, in the scale, fresh fillets, and culturing water of tilapia (Al-Harbi and Uddin, 2004; Boari *et al*., 2008). *Staphylococcus* spp. and *Macrococcus caseolyticus* have a similar morphology and closed generic relationship. Bacterial phenotypes of them are coccus-shaped, non-motile, non-spore forming, and without granules in cell. However, two species have different percentages of genomic content; *Macrococcus* was reported to have DNA G+C content higher than 38-45 (Kloos *et al*., 1998), while, *Staphylococcus* had 33-40 (Endl *et al*., 1983). They severely caused in codfish mortality (Vilhelmsson *et al*., 1997) and it has been used as bacterial pathogen in marine catfish (Pandey *et al*., 2010). But these strains were inhibited *A. hydrophila*.

The study of adhesive potential *in vitro* assays is every important. The prerequisite of potential probiotics is their colonization within the host's GIT, which in turn, is dependent upon the ability of the species to adhere to the host-cells and/or mucus. The adhesive ability of probiotic candidates is considered to associate for colonizing to the intestinal tract of fish (Ringø and Gatesoupe, 1998; Ouwehand and Salminen, 2003). The viable adhesive potential of *Bacillus* probiotic candidates has been reported that ranging 0.001 to 0.305% for estimating number of bacterial cells per epithelial cells (Prieto *et al*., 2014). The ability of lactic acid bacteria (LAB) to adhere to the intestine mucus has found from 16 to 20% (Balcázar *et al*., 2007). In this study, the average adhesion of all incubation times of bacterial isolates to the intestinal cells of tilapia was found variable between 1 to 13%. The highest adhesive-potential to tilapia intestinal cells was found in *Bacillus* spp. CHP02.

Adhesion to hydrocarbons as a simple method evaluates the ability of bacteria to adhere to non-specific surface (Rosenberg *et al.*, 1985), which is used a means to assess the potential of probiotic candidates to adhere intestinal mucusa (Otero *et al.*, 2004). However, a variable adhesive between bacterial adhesion rates to hydrocarbons (n-hexadecane, xylene and toluene) has been reported to be both species and hydrocarbon specific, with LAB displayed 6 to 73 % (Dhewa *et al*., 2009), bacterial isolates form shrimp farming displayed 15 to 70% adhesion to *P*-xylene, ethyl acetate, and chloroform (Sánchez-Ortiz *et al*., 2015) and autochthonous *Bacillus infantis* form *Labeo rohita* displayed 9 to 24% adhesion to hydrocarbons (xylene, ethyl acetate, and chloroform) (Dharmaraj and Rajendren, 2014). Bacteria cells contain many molecules underpin the morphology, polarity and biochemical properties of the cell, which influence the

degree of adhesion hydrocarbons (Sánchez-Ortiz *et al*., 2015). In this study, we also found vary potentials to adhere for hydrocarbons of isolates and the highest adhesive-potential for hydrocarbons only was found in *Enterobactor* sp. displayed 95% to chloroform and 49% to hexane. Low adhesions in hexane might be affected by its strong organic solvent for bacteria, which indicated using the absorbance at the end of the incubation period.

An early aggregation of bacteria could provide mass number to colonize to the mucosal surfaces of the host (Grześkowiak *et al*., 2012), which report 1 to 70% of auto-aggregated abilities (Kos *et al*., 2003; Pan *et al*., 2008; Lazado *et al*., 2011; Abdulla *et al*., 2014). This study, the ability of isolates to adhere cells-to-cells of the same strain, was evaluated this potential in buffer solutions (PBS and 0.85% NaCl), which found ranging 2 to 70% auto-aggregation. A high potential was found in *Bacillus* sp. RC02 displayed a high aggregation in both PBS and 0.85% NaCl. We clearly showed that isolates have varying potentials to adhere to different assays.

Probiotic candidates have been reported to show resistances to a number of different antibiotics (Mourad and Nour-Eddine, 2006; Liasi *et al*., 2009; Nayak and Mukherjee, 2011). This study was found variable antibiotic susceptibilities of these isolates. The multi-antibiotic resistance was found in *Bacillus* sp. CHP01 to resist to sulphamethoxazole/thrimethoprim, ampicillin, and cephalothin. It was an interested point, which four isolates (*Bacillus* CHP00 CHP01, *Mac. caseolyticus* CHP03 and *Stap. arletae* CHP04) originated from offspring tilapia in the closed system using tap water. They displayed to antibiotic resistances. The possible reason may be related to current practices in many farms in Asia (tilapia farm, shrimp culture and pangasius farms) using high levels of antibiotics such as enrofloxacin, chloramphenicol, sulfadiazine, and

trimethoprim (Alday *et al*., 2006; Rico *et al*., 2013). These residual antibiotics may lead, bacteria to resist antibiotic drugs and bacteria containing resist genes can inherit from generation to generation. FAO/WTO, (2006) advocate probiotics should need the clarified determination of safety parameters such as lack of antibiotic resistance and virulence genes to lyse erythrocytes. Thus, bacteria contain virulence genes to blood hemolytic activities, which revealed to harmful bacteria (Scheffer *et al*., 1988). These bacteria can express to positive on blood agar plates *in vitro* assay. We found *Bacillus* sp. RC02 and all *B. cereus* strains (CHP00, NP00 and NP01) displayed β-hemolysis on blood agar plates. *B. cereus* is a known human pathogen (Ceuppens *et al*., 2013).

Potential probiotics need to be able tolerate to pH and bile salt stimulations. Hlophe *et al*., (2013) reported that pH of Nile tilapia stomach varies 1.6 to 5.0 and bile concentrations in salmon fish has estimated ranging from 0.4 to 1.3% (Balcázar *et al*., 2008). Several studies have reported that probiotics could tolerate to pH values 1 to 12 and 2 to 12% bile salts (Mourad and Nour-Eddine, 2006; Balcázar *et al*., 2008; Nayak and Mukherjee, 2011; Chemlal-Kherraz *et al*., 2012; Geraylou *et al*., 2014). Most studies reported that probiotic candidates could display resistance to pH 2. The result of the current study, we evaluated potential capacities of isolates to low pH (24 hours) and found all *Bacillus* strains tolerated at pH 2. All isolates tolerated 6% bile salts and five isolates tolerated to 12% bile salts. The possibility of tolerance to low pH of probiotic candidates might be important than bile salts.

Fish are poikilothermic, thus temperature has a great affect on the bacterial GIT growth, auto-aggregation/adhesion, and species diversity (Ibrahim *et al*., 2004; Collado *et al*., 2008; Kosin

and Rakshit, 2010; Rahiman *et al*., 2010; Nayak and Mukherjee, 2011). The change of isolates at different temperatures as un-optimal and optimal conditions for tilapia culture was assessed in the present study. All isolates had high capacities to grow at temperatures more than 32^{o}C, whilst, at low temperature seemed to be effect on bacterial differences. Four isolates: *Bacillus* sp. CHP01, *Stap. sciuri* NP04, *Bacillus* sp. RP01 and *Enterobacter* sp. NP03 were dominant at low temperature (15^{O}C). We can suggest that temperature changes might affect a putative number of the intestinal bacteria for tilapia culture.

Furthermore, antagonistic activities are the popular criterion to simply for probiotic selection (Lauzon *et al*., 2008; Das *et al*., 2013; Liu *et al*., 2013). The ranking index by using growth properties *in vitro* testing has been reported to use as the criterion to select probiotics (Vine *et al*., 2004). Balcázar *et al*., (2008&2016) used pH and bile salt tolerances, adhesion to fish mucus and pathogenic inhibition for selecting probiotics, and Grześkowiak *et al*., (2012) used abilities of auto-aggregation and co-aggregation. Earlier reported has suggested that hydrophobicity values having than 40% could be suitably used for probiotic selection (Abdulla *et al*., 2014). According to, several articles used many parameters *in vitro* trials for selecting potential probiotics, which found different results (Balcázar *et al*., 2007&2008; Chemlal-Kherraz *et al*., 2012). At the same of results in this study, then, findings of multi-parameter were combined together for selecting probiotic candidates, which provided as the Z−score method. High potentials of probiotic candidates were found in *Bacillus* sp. CHP02, *Bacillus* sp. RP01, and *Bacillus* sp. RP00. Several isolates consisted of *Stap. arlettae* CHP04, *Enterobacter* sp. NP03, *B. cereus* NP01, *B. cereus* CHP00, *Bacillus* sp. RC02, *B. cereus* NP00, and *Bacillus*

sp. CHP01 had minus Z−scores, which expressed antibiotic resistances and positive blood hemolytic activities.

In conclusion, the combined selection using the Z−scores calculation might be used to select high potential probiotic candidates. The multi-parameter *in vitro* assays in the present study, parameters consisting of pathogen antagonism, adhesion assays, auto-aggregations, potentials to tolerate with pH and bile salt concentrations and bacterial changes at temperature exposures, were used to combination for selecting potential probiotics. The highest ranked potential candidate was *Bacillus* sp. CHP02. This strain displays many favorable properties: (i) inhibition to pathogens, (ii) high adhesive potential to the tilapia epithelial cells, (iii) adhesive potential for hydrocarbons, (iv) auto-aggregations, (v) an antibiotic susceptibility, (vi) non-hemolytic activity, (vii) tolerance to 6% bile salts, (viii) resistance to pH 2, and (ix) acceptable growth at temperatures approve to tilapia farming. This strain, and other high scoring isolates will be tested *in vivo* in Chapter 4 and 5 to ascertain probiotic efficacy and to determine if the Z-score ranking approach is a valid tool for selecting favorable probiotic candidates.

Chapter 4

In vivo trial using tilapia larvae

4.1 Abstract

Tilapia larvae were fed with one of six different commercial diets containing potential probiotics at 10^{6-7} cfu.g^{-1}: T1: (*Bacillus* sp. CHP02), T2: (*Bacillus* sp. RP01), T3: (*Bacillus* sp. RP00), T4: (*Enterobacter* sp. NP02), T5: (*P. acidilactici*) or T6: (control group – no probiotic). One thousand eight hundred tilapia larvae (8.1±0.8 mg) were organized into triplicate containers for each experimental group. Samples were reared in the containers for 6 weeks. At the end of the trial, significant differences (P<0.05) of average body weight, total weight gain, average daily growth, and specific growth rate were observed between the treatment groups. The T1 group displayed the highest body weight more than the other groups and the lower body weight were found in the T5 and T6 groups. The weight gain, average daily growth, and specific growth rate were significant higher in the T2 group more than the other groups and the lower of these parameters were found in the T5 and T6 groups. No significant differences (P>0.05) among treatments were found in parameters of length gain, K factors, RIL, survival rate, levels of cultivable microbes in the intestine (log cfu.g^{-1}), the density of goblet cells, the proportion of microvilli length per width and microvilli area were observed. *Bacillus* were detected variable samples in treatment studies both the trial mid point and the end of the trial. Only the T1 group was observed *Bacillus* to colonize in all samples. At the end of the feeding-trial fish were challenged by *A. hydrophila*. Probiotic diets displayed significantly (P<0.05) improved survival (93 −100%) against *A. hydrophila* after 7 days of IP challenge more than the control group (76%). Collectively, these results indicate that *Bacillus* sp. RP01 has positive effects on tilapia larvae including improved body weight, total weight gain, average daily growth, specific growth rate and resistance to *A. hydrophila* challenge.

4.2 Introduction

Tilapia farmers require large numbers of larvae with high qualities of growth performance, disease resistance and high survival rate. At the initial stage of larval feeding, different sizes of feed are required. The survival rate of tilapia through the larval stage has been reported to be as low as approximately 60% (Boyd, 2004). It is therefore important to maintain biosecurity to support high survival rate of tilapia larvae in hatcheries. However, a sterile environment in the hatchery may also lead to poor growth in grow-out farms (Gomes-Gil *et al*., 2000). Because of larval microbes may associate microbial translocations of exogenous pathogenic and beneficial bacteria, which may adhere in the digestive tract (Ringø *et al*., 2007; Giatsis *et al*., 2014). Probiotics, as a means for microbial control, have been reported to improve growth performances and survival rates of tilapia (Lara-Flores *et al*., 2003; Apún-Molina *et al*., 2009; He *et al*., 2013). In addition, it has been reported that probiotics support good growth performances of tilapia larvae fed a low protein diet (Ghazalah *et al*., 2010). Several researchers have published potential probiotics in tilapia larvae after reversing to male phenotypes having 0.1 to 5 g of body weight (Lara-Flores *et al*., 2003; Shelby *et al*., 2006; Apún-Molina *et al*., 2009; Ali *et al*., 2010; Liu *et al*., 2013; He *et al*., 2013). Then, in this study we evaluated the potential of probiotic candidates on the early larvae without sex-reversal of tilapia (total weight of 7 to 9 mg).

Given the importance of the larval stage in the life cycle, and the relative lack of probiotic research on larval tilapia, the aims of this study were to evaluate the potential of probiotic candidates derived from Chapter 3 on tilapia fry at the initial feeding stage by observing growth performances, bacterial counts in the tilapia intestine, intestinal histological parameters and disease resistance. The highest-ranking autochthonous potential probiotics according to the Z−score calculations from chapter 3 were *Bacillus* sp. CHP02 (1.14), *Bacillus* sp. RP01 (1.09), *Bacillus* sp. RP00 (0.94), *Bacillus* sp. RC01 (0.83), *Stap. sciuri* NP04 (0.63), *Bacillus* sp. RC00 (0.61), *Enterobactor* sp.

NP02 (0.50), *Stap. arlettae* CHP04 (0.45) and *Mac. caseolyticus* CHP03 (0.32). The limitation of facilities, top three ranking of *Bacillus* spp. CHP02, RP01 & RP00 and *Enterobactor* sp. NP02 (Appendix 1) were selected for these *in vivo* studies. In addition, the well-documented probiotic *P. acidilactici* was also investigated as a reference strain.

4.3 Materials and methods

4.3.1 Fry tilapia preparation

The early swim-up fry of tilapia (*O. niloticus*) at 4-5 day post-hatch (dph) were provided by AIT, Thailand. These larvae were transferred to KMITL within an hour for acclimating in running water system for two days (Figure 4.1). At 7 dph fry, without sex reversion had a mean weight of 0.0081±0.0008 g and mean length of 0.87±0.05 cm.

4.3.2 Experimental trial

A total of 1,800 larvae (7 dph) were used in six experiments having triplicate containers per treatment. These treatments were randomly assigned to separate cement ponds. One hundred larval fish were randomly distributed into the container (13 l) suspending in cement ponds (508 l) with aeration and flow-through water (2.5 $l.min^{-1}$). Larvae were fed one of six different commercial diets containing potential probiotics at 10^{6-7} $cfu.g^{-1}$: T1: (*Bacillus* sp. CHP02), T2: (*Bacillus* sp. RP01), T3: (*Bacillus* sp. RP00), T4: (*Enterobacter* sp. NP02), T5: (*P. acidilactici*) or T6: (control group – no probiotic). The probiotics and fish feed were prepared as described in section **2.4**. These fish were fed six days a week to apparent satiation five times a day (every 2 hours from 9.00AM to 5.00PM). Fish excreta of every pond were drained twice per week. During the experiment, dead fish were daily recorded and removed from containers.

Water quality was monitored weekly during the experiment. These were as follows: 30.3±0.1^{O}C for water temperature, 5.60±0.36 mg.l^{-1} for dissolved oxygen, 7.0±0.0 for pH and 0.38±0.05 mg–N.l^{-1} for TAN.

4.3.3 Growth parameters

During the six weeks of feeding trials, the body weight and the total length of fry samples were obtained weekly (as described in section **2.5**). The average body weight was determined every week. Parameters of WG, TLG, ADG, SGR, K factors, the RIL were determined at the trial mid point (3 weeks) and the trial ending (6 weeks). The SR was determined at the trial ending. These parameters were calculated as explains in **2.5.1**.

Figure 4.1 Acclimation of tilapia larvae in the rearing system.

4.3.4 Bacterial studies

4.3.4.1 Plating and colony counts

The estimation of bacterial loads in the intestine of samples was determined at the middle and the end of the trial. Fish were deprived of feed for 24 hours, and the intestines of nine fish of each treatment (three fish per replicate container) was removed (Figure 4.2: as described in **2.2**) to enumerate microbial loads. The GI solution of an individual sample (Figure 2.1: part 4) was used estimation of viable microbial count by using serial dilutions (as explained in **2.3.1**). A total volume of 100 µL of 10^{-1}, $10^{-3\ to\ -4}$, $10^{-3\ to\ -4}$ and $10^{-7\ to\ -8}$ were spread onto duplicate MRS-A, EMB (Himedia, India), BA medium and TSA, respectively. Agar plates were incubated at 32 ^{0}C for 48 hours to record photographs and then the ImageJ 1.48v software (national Institutes of Health, USA) was used to manually count microbial loads (cfu.g^{-1}) in each sample.

Figure 4.2 The GIT of an individual larval tilapia was removed under aseptic and cool conditions.

4.3.4.2 Probiotic monitoring

Three were designed to monitor probiotics colonized in the GIT. Triplicated intestine in each replicate (Figure 2.1: part 3) were homogenized in ASL buffer and then samples were centrifuged to remove supernatant to mix with Inhibit EX tablet. Samples were centrifuged to remove supernatant and then added Proteinase K and AL buffer in samples. Samples were incubation and added absolute ethanol. Finally, samples were washed with AW1 and AW2 buffers. Genomic bacteria were maintained in AE buffer. The process of genomic DNA extraction was described in ***2.3.5.1*** (QIAamp DNA Stool Mini Kit, Qiagen). These samples were monitored probiotics as *Bacillus* spp., *Enterobactor* sp. and *P. acidilactici* to colonise in the GIT by using specific probiotic primers (Table 2.1). The genomic DNA of each replicate was pooled into a single sample. The total volume of PCR was 25 µL: 12.5 µL of the GoTaq® Green Master Mix, 2.5 µl of 10 µM of each primer, 1 µL of DNA template and 6.5 µL of sterile distilled water. The cycling conditions were depended on different probiotic primers, which explained in ***2.3.5.2***. The PCR products targeting of primer synthesis were checked by using agarose gels 1.5% (w/v) containing RedSafe DNA Stain (0.005 %) as explained in ***2.3.5.3***. Document gels were interpreted comparing with positive probiotic bands.

4.3.5 Microscopic studies

At the trial mid-point and the trial ending, three samples of each treatment were used the mid-intestinal tract (Figure 2.1: part 1) to study intestinal morphology and the density of goblet cells by using LM. Samples were prepared as described in ***2.5.3.1***. These samples were counted goblet cells using ImageJ 1.48v software (National Institutes of Health, USA) and then the density of goblet cells was calculated (cell/0.1mm^2).

At the mid-trial and the trial ending, three samples of each treatment were taken to estimate microvilli length and width using TEM (Phillips: Techni20, Holland). The mid-intestinal sample

(Figure 2.1: part 2) was prepared as explained in ***2.5.5.2***. The ImageJ 1.48v software (National Institutes of Health, USA) was used to measure microvilli length (h_{mi}) and microvilli width (w_{mi}) from the micrographs. Microvilli areas of samples were calculated by using the equation of $2\pi rh+\pi r^2$ (r=radian of microvilli; $w_{mi}/2$, and h=microvilli length; h_{mi} as Figure 2.5).

At the mid-trial and the trial ending, SEM was used to monitor microbial colonizing in GI tract. Three pieces of the mid-intestinal sample (Figure 2.1: part 2) of each replicate were prepared as described in ***2.5.3.3***. These samples were dehydrated and coated gold (Cressington Sputter Coater, 108 auto). Samples were scanned and imaged to assess the microbial colonization on the intestinal epithelial cells using a SEM (Carl Zeiss: EVO® HD, USA).

4.3.6 Disease resistance

At the ending of the trial, 25 fish from each container were injected with 0.1 ml *A. hydrophila* (1×10^{10} cfu.ml^{-1}) into the IP cavity of the fish. *A. hydrophila* were supplied by AAHRI, Thailand, which were activated as ***3.3.2.1***. In addition, 25 residual fish were randomized to inject 0.1 ml of sterile 0.85% NaCl. These fish were kept separately in a container for 7 days to monitor fish mortality.

4.3.7 Statistical analysis

The findings were displayed in terms of mean ± standard deviation. Percentage data recordings and viable counts were transformed to normality. Growth performances, log viable counts, and other parameters compared by using a one-way analysis of variance (ANOVA). Significant differences between groups were accepted at $P< 0.05$. Pairwise comparison probabilities used to compare difference among means of treatments. The Systat software ver. 5.02 (Illinois, USA) was used to analyze these data.

4.4 Results

4.4.1 Growth performance

The average body weight (g) of all treatments in each week is displayed in Table 4.1. The significant difference (P<0.05) among treatments was initially observed in the first week, which displayed the highest body weight in the control group than probiotic groups. However, high average body weights at the ending of this trial were observed in probiotic groups than the control group. The highest was found in T2 samples fed *Bacillus* BRP01 mixing in feed. At the mid-trial, significant differences (P<0.05) between treatments of WG, ADG, and SGR were found and no differences (P>0.05) in parameters of TLG, K, and RIL were found (Table 4.2). At the trial ending, significant differences (P<0.05) between treatments of WG, ADG, and SGR were found and no differences (P>0.05) in the parameters of TLG, K, and RIL were found (Table 4.3). The most efficiency on growth performances was found in T2 treatment, which displayed to be 70496.26±1321.31 of WG, 0.14±0.00 of ADG and 6.78±0.02 of SGR. The survival rate (Figure 4.3) of experimental groups showed not significant (P>0.05), which had approximately seventy-five percent (74±5).

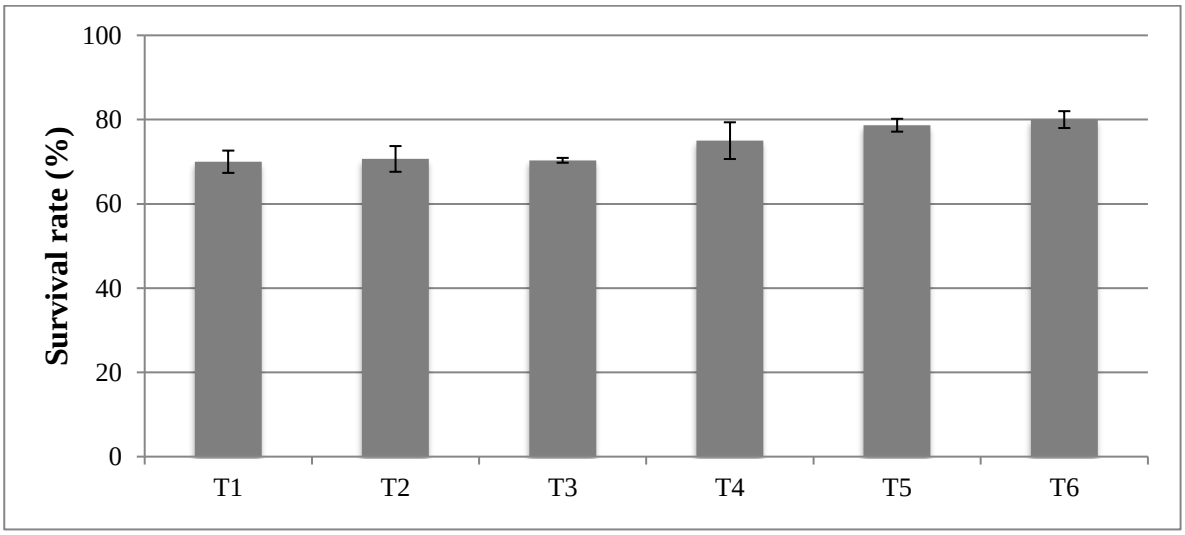


Figure 4.3 The survival rate (mean and standard error) of tilapia larvae fed different dietary treatments.

Table 4.1 Average wet weight (g) of different treatments in each week of experimental feeding.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
T1	0.038±0.007^{ab}	0.239±0.051^{ab}	0.663±0.139^{b}	1.190±0.174^{b}	3.795±0.786^{b}	3.998±1.180^{b}
T2	0.037±0.005^{ab}	0.253±0.024^{a}	0.795±0.166^{a}	1.535±0.186^{a}	4.456±0.855^{a}	5.718±1.292^{a}
T3	0.036±0.005^{a}	0.212±0.031^{ab}	0.648±0.117^{c}	1.186±0.170^{b}	3.874±1.105^{b}	4.125±1.432^{b}
T4	0.039±0.005^{ab}	0.201±0.0252 ^{ab}	0.576±0.141^{cd}	1.105±0.142^{b}	2.819±0.888^{c}	3.939±1.227^{b}
T5	0.038±0.006^{ab}	0.200±0.045 ^{ab}	0.569±0.127^{cd}	1.020±0.125^{b}	2.306±0.903^{cd}	3.078±1.404^{c}
T6	0.043±0.008^{b}	0.202±0.030^{b}	0.540±0.090^{d}	1.029±0.172^{b}	2.068±0.759^{d}	2.868±1.105^{c}

Presented values are means of triplicates ± standard error of mean and denoted significant differences (P<0.05) between treatments in each week by using different superscripts in each column.

Table 4.2 *In vivo* trial mid point growth performance data.

Parameters	Treatments					
	T1	T2	T3	T4	T5	T6
WG, %	8081.03±514.91^{b}	9716.48±107.06^{a}	7894.37±385.95^{b}	7014.74±377.68^{b}	6919.97±77.72^{c}	6563.81±62.97^{c}
TLG, %	307.85±15.89	318.89±9.81	291.32±27.43	290.95±5.56	275.42±36.74	271.78±9.77
ADG	0.03±0.00^{b}	0.04±0.00^{a}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{b}
SGR, %	9.106±0.132^{b}	9.485±0.023^{a}	9.059±0.099^{b}	8.818±0.112^{b}	8.792±0.023^{c}	8.684±0.020^{c}
K	1.488±0.143	1.645±0.097	1.662±0.267	1.464±0.066	1.701±0.536	1.600±0.137
RIL	2.74±0.44	3.13±0.62	2.98±0.59	2.93±0.40	2.91±0.40	3.06±0.48

Presented values are means of triplicates ± standard error of mean and denoted significant differences (P<0.05) between treatments in each week by using different superscripts in each column.

Table 4.3 *In vivo* trial end point growth performance data.

Parameters	Treatments					
	T1	**T2**	**T3**	**T4**	**T5**	**T6**
WG, %	49261.12±1979.53^{b}	70496.26±1321.3^{a}	50825.50±4466.56^{b}	48525.62±1813.65^{b}	37899.49±564.54^{c}	35302.34±1399.83^{c}
TLG, %	556.26±66.57	658.01±31.43	609.81±28.19	596.56±8.89	577.00±20.05	558.60±27.18
ADG	0.10±0.00^{b}	0.14±0.00^{a}	0.10±0.01^{b}	0.09±0.00^{b}	0.07±0.00^{c}	0.07±0.00^{c}
SGR, %	6.412±0.042^{b}	6.783±0.019^{a}	6.442±0.093^{b}	6.397±0.038^{b}	6.142±0.015^{c}	6.069±0.041^{c}
K	2.249±0.765	2.011±0.298	1.774±0.349	1.772±0.124	1.511±0.123	1.539±0.234
RIL	4.08±0.84	4.59±0.59	4.64±0.39	4.50±0.35	4.13±0.35	4.00±0.45

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

4.4.2 The microbial intestinal count and probiotic monitoring in larval tilapia

Cultivable microbes in GI tract of tilapia larvae (0.008±0.001 g; N=25) on EMA, BA and TSA were 3.6 to 3.9, 3.8 to 4.0 and 4.1 to 4.2 log cfu.g^{-1}, respectively. At the mid-trial, both probiotic groups (T1 to T5) and the control group (T6) were displayed a few number of microbial cells on MRS agar plates and microbial number were increased at the end of the trial (Table 4.4). The comparison of the intestinal microbes on the same medium of different treatments at the mid and the trial endings were found no significant differences (Table 4.4). However, the highest abundance of the intestinal microbes on TSA medium at the end of the trial was found in the T1 group than other groups. We observed usually fungi and yeast occurring on MRS-A medium. The number of microbial loads in the intestine both Gram-negative bacteria on EMA and Gram-positive bacilli on BA tended to be increasing time studies.

Table 4.4 Mean and standard error of cultivable microbial loads (log cfu.g^{-1}) in the tilapia intestine of different treatments observed on different media.

Treatments	MRS-A		EMA		BA		TSA	
	Week 3	Week 6	Week 3	Week 6	Week 3	Week 6	Week 3	Week 6
T1	nd	2.48±0.59	6.46±0.26	6.49±0.23	4.63±0.35	6.49±0.37	5.29±0.28	8.58±0.36
T2	nd	2.23±0.44	5.19±0.80	6.24±0.05	5.31±0.11	6.24±0.07	5.58±0.06	7.22±0.05
T3	0.2	1.84±0.07	4.60±0.52	7.10±0.93	5.29±0.42	6.92±0.06	5.29±0.34	7.47±0.26
T4	0.4	2.38±0.25	4.02±0.27	6.72±0.66	4.62±0.27	7.13±0.85	5.15±0.08	7.72±0.67
T5	nd	1.89±0.37	3.79±0.26	6.48±0.04	4.33±0.37	6.49±0.03	4.82±0.55	7.76±0.39
T6	nd	2.42±0.18	4.72±0.83	7.06±0.87	4.74±0.93	6.51±0.12	5.35±0.64	7.60±0.27

nd = not detected

After the mid-trial, samples of all treatment diets were presented *Bacillus* in the GIT of tilapia larvae (Figure 4.4). The T1 and T5 groups were observed *Bacillus* to colonize in all samples. *Enterobacter* sp. and *P. acidilactici* probiotics were not detected in the GIT of any of the treatments.

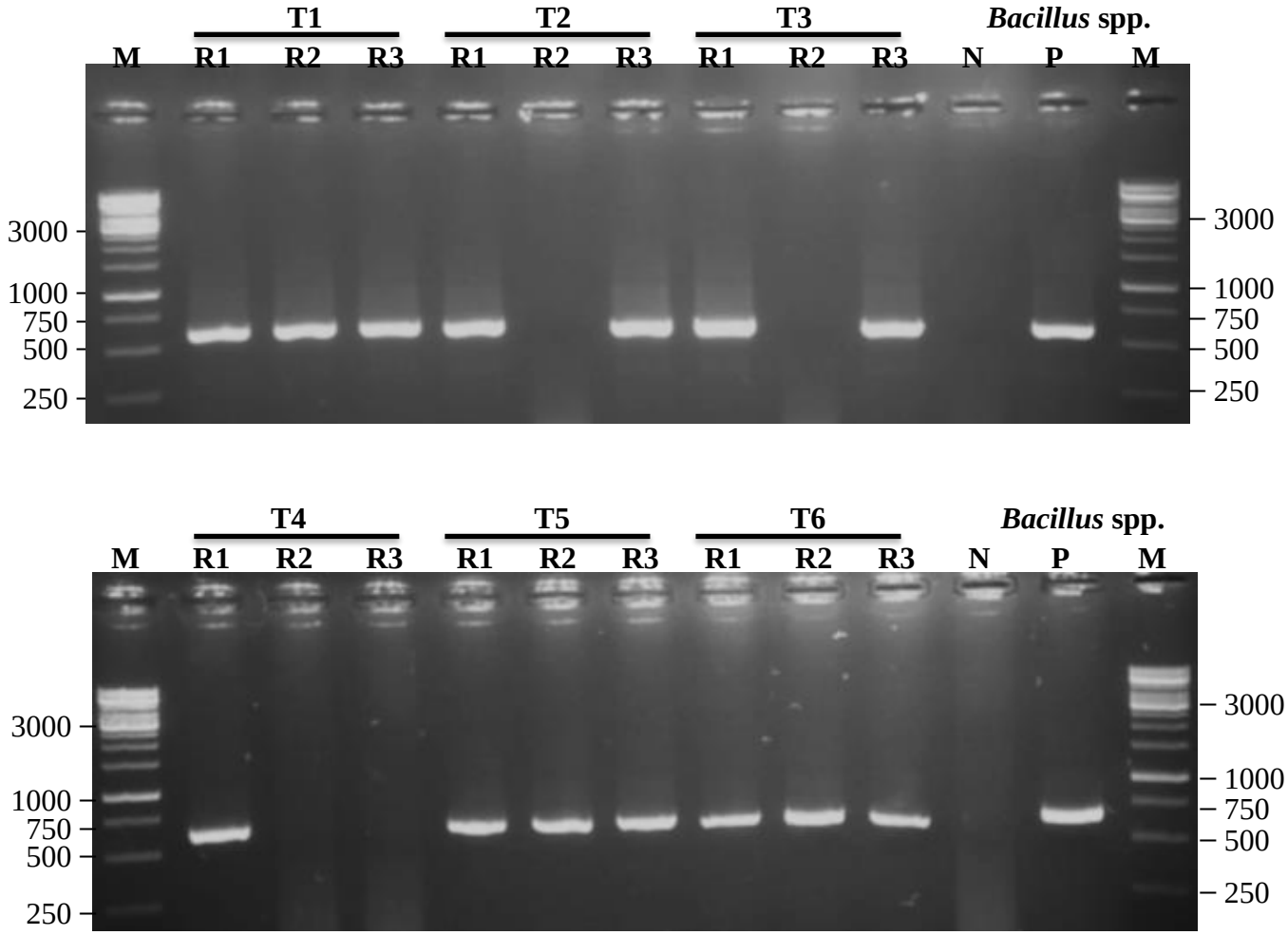


Figure 4.4 Probiotic monitoring using *Bacillus* primer to detect probiotic colonization in the larval intestine at 3 weeks (M=100 bp plus DNA marker (Fermentas); N=Negative control (pure sterile water used as DNA template) and P=Positive control (Positive probiotics as used probiotic DNA templates); T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group).

At the end of the trial, the T1 and T3 groups were detected *Bacillus* to colonize in the GIT of tilapia larvae. A few samples of T2, T4 and T6 groups were also observed *Bacillus* and only the T5 group displayed non-colonisation of *Bacillus* (Figure 4.5). Both *Enterobacter* sp. and *P. acidilactici* probiotics were observed non-colonisation in the GIT of treatments.

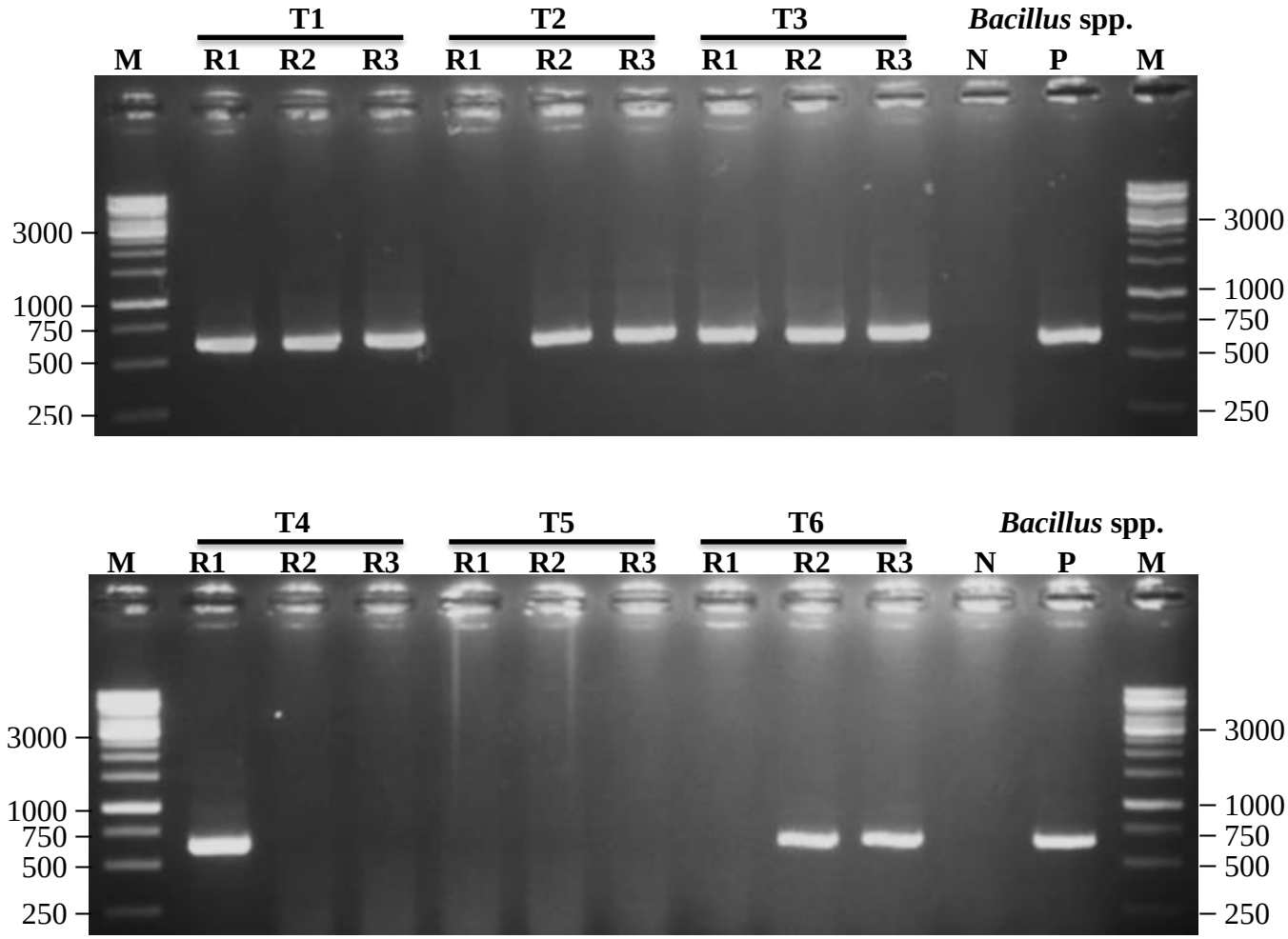


Figure 4.5 Probiotic monitoring using *Bacillus* primer to detect probiotic colonization in the larval intestine at 6 weeks (M=100 bp plus DNA marker (Fermentas); N=Negative control (pure sterile water used as DNA template) and P=Positive control (Positive probiotics as used probiotic DNA templates); T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group).

4.4.3 Microscopic studies

At the mid-trial and the end of the trial, the intestinal morphology of tilapia larvae fed each of different diets was examined by light microscopy (Figure 4.6 & 4.7). A simple columnar epithelium with mucosal folds were extended into the intestinal lumen was observed in samples. Each mucosal fold consisted of lamina propria, surrounded by a polarised layer of enterocytes interspersed by goblet cells and intraepithelial leucocytes. No significant differences of the abundance of goblet cells between treatments at the mid-trial and the end of the trial were observed ($P > 0.05$) (Figure 4.8).

TEM micrographs were used to assess the morphology of the intestinal microvilli at the mid point (Figure 4.9) and the end point of the trial (Figure 4.10). Observations revealed well-formed, long, intact microvilli on the apical surfaces of enterocytes from all treatment groups. The microvilli parameters of length, width, the proportion of length/width, and microvilli area were observed no significant-differences between the groups at the mid-trial (Table 4.4). At the end of the trial, microvilli length and width (Table 4.4) were observed significant differences ($P<0.05$). The microvilli length in T1, T2, T4, T5 and T6 were higher than T3. The microvilli width in T1, T4 and T5 were differently higher than T1.

SEM micrographs (Figures 4.11 & 4.12) revealed complex mucosal folds and packed microvilli on the apical surfaces, with minor residues of mucus and digesta. Bacteria-like cells were also observed adhering to the mucosal epithelium, which were presumably autochthonous bacteria of the tilapia intestine. Several bacterial phenotypes (rod-shape and cocci-shape) were observed but no qualitative changes in abundance or colonization patterns were observed.

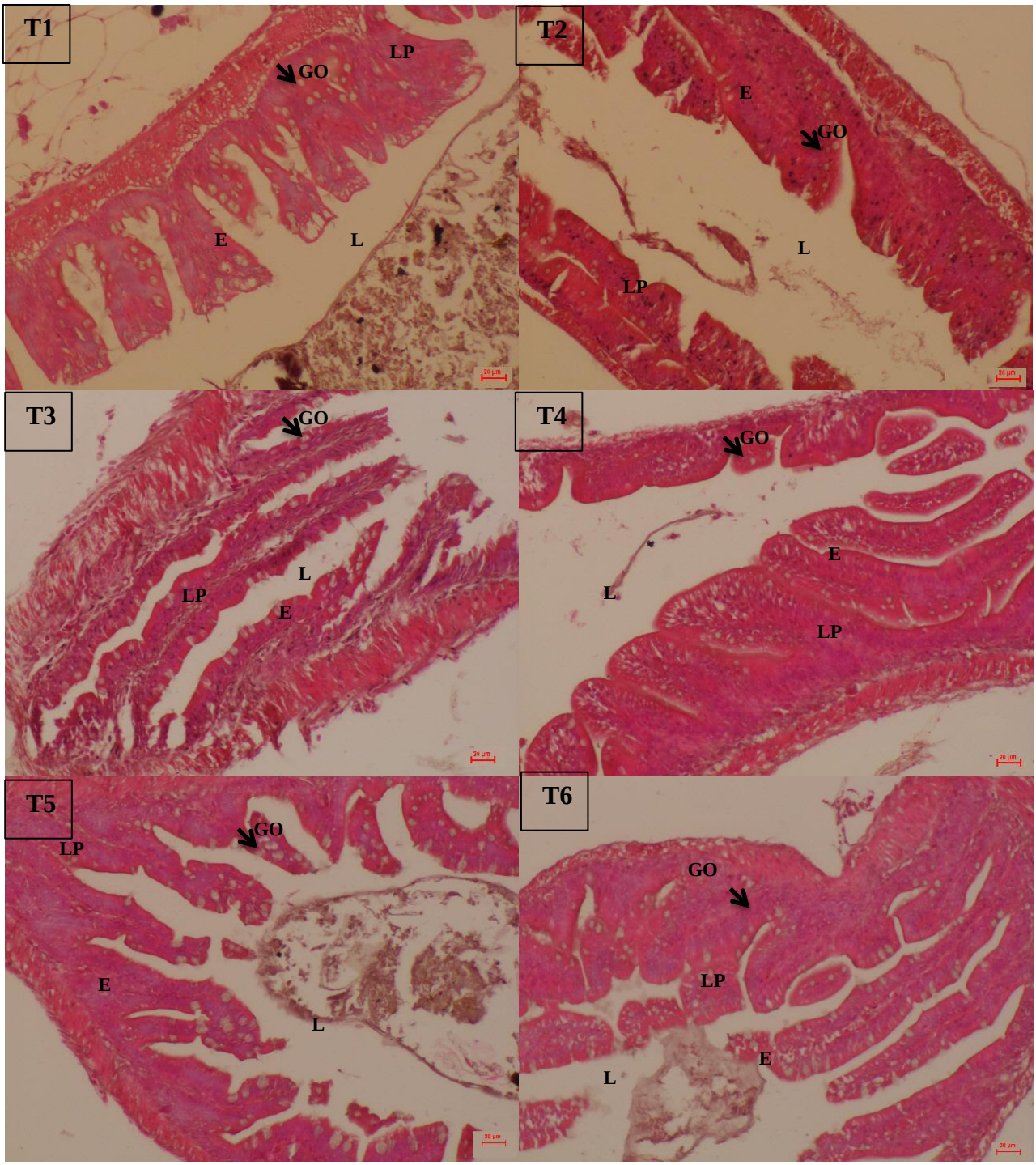


Figure 4.6 Light micrographs of the mid-intestine (H&E staining) of tilapia in different groups after feeding probiotic at 3 weeks (L=lumen, LP= lumina propria, E=epithelia, GO=goblet cells; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=20 μm.

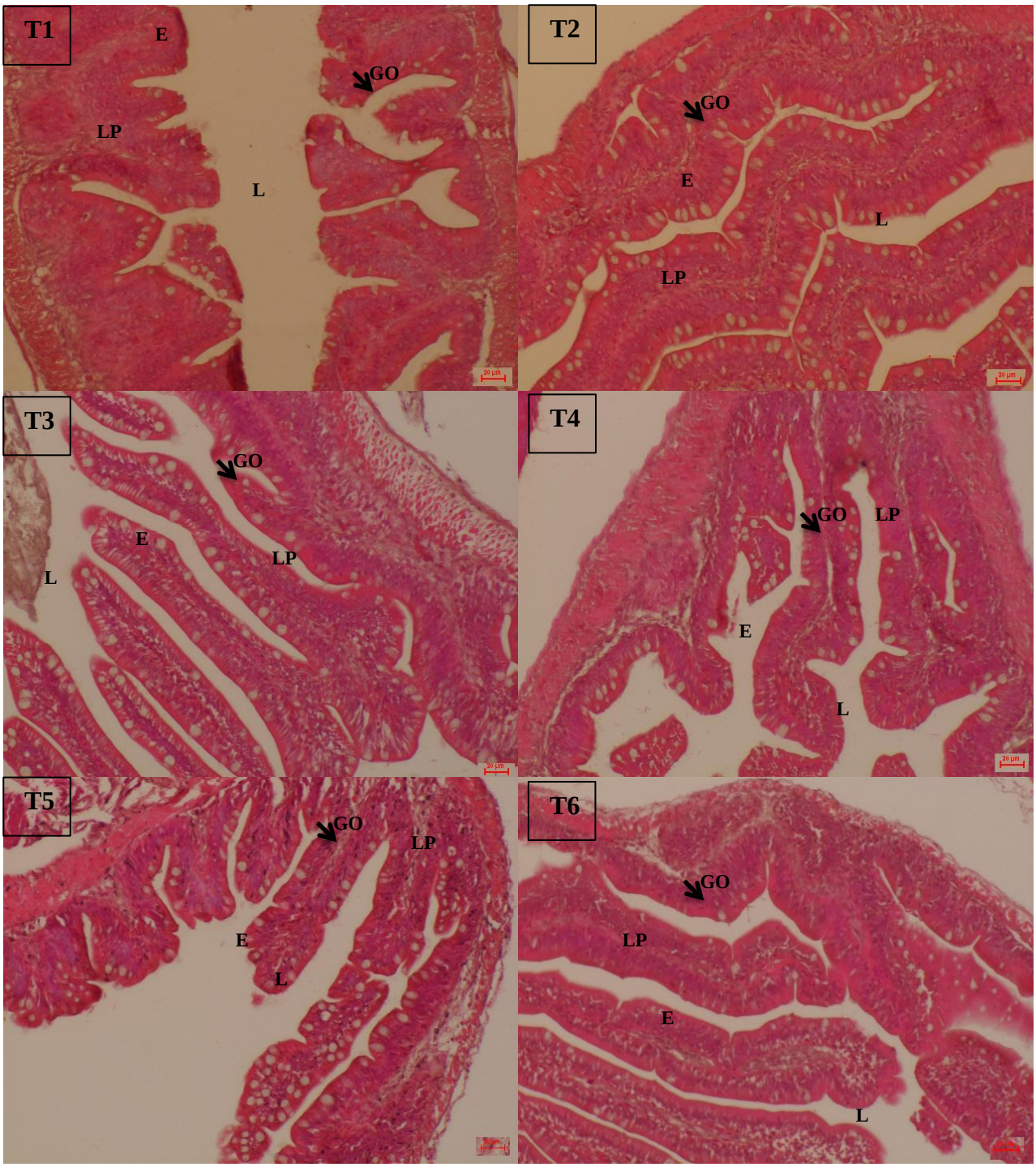


Figure 4.7 Light micrographs of the mid-intestine (H&E staining) of tilapia in different groups after feeding probiotic at 6 weeks (L=lumen, LP= lumina propria, E=epithelia, GO=goblet cells; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=20 μm.

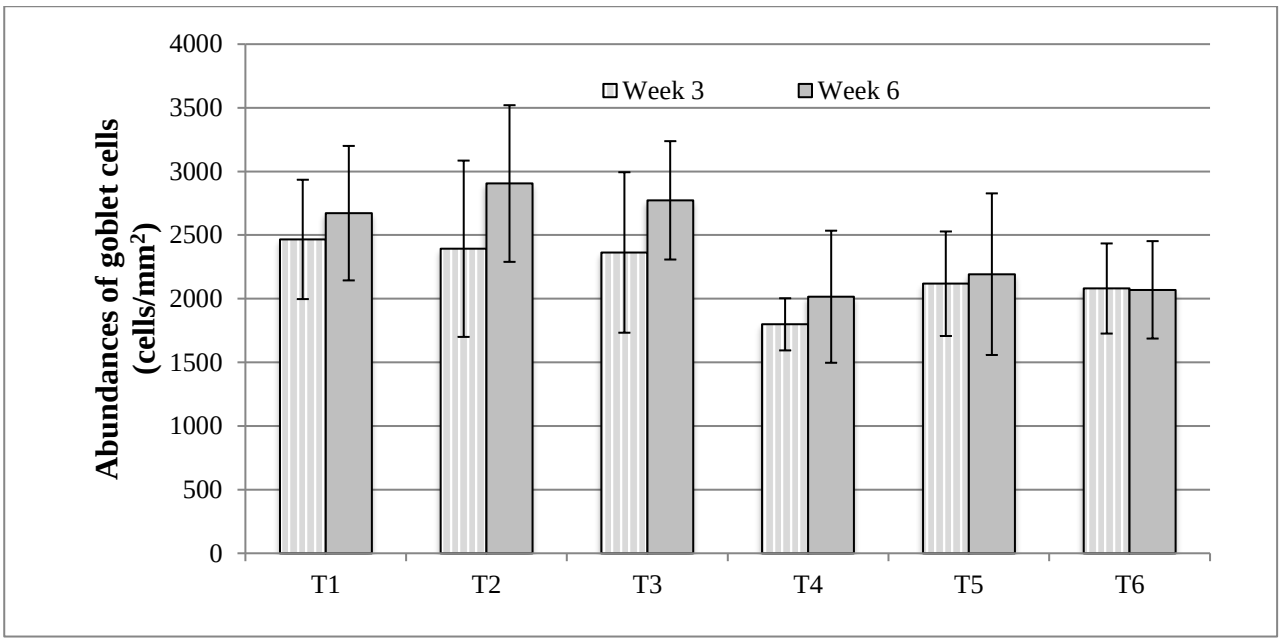


Figure 4.8 Abundances of goblet cells (mean and standard error) fed of different treatments at the mid-trial (3 weeks) and the trial ending (6 weeks). Presented values are means of triplicates ± standard error of mean and denoted non-significant differences (P>0.05) between treatments in each week.

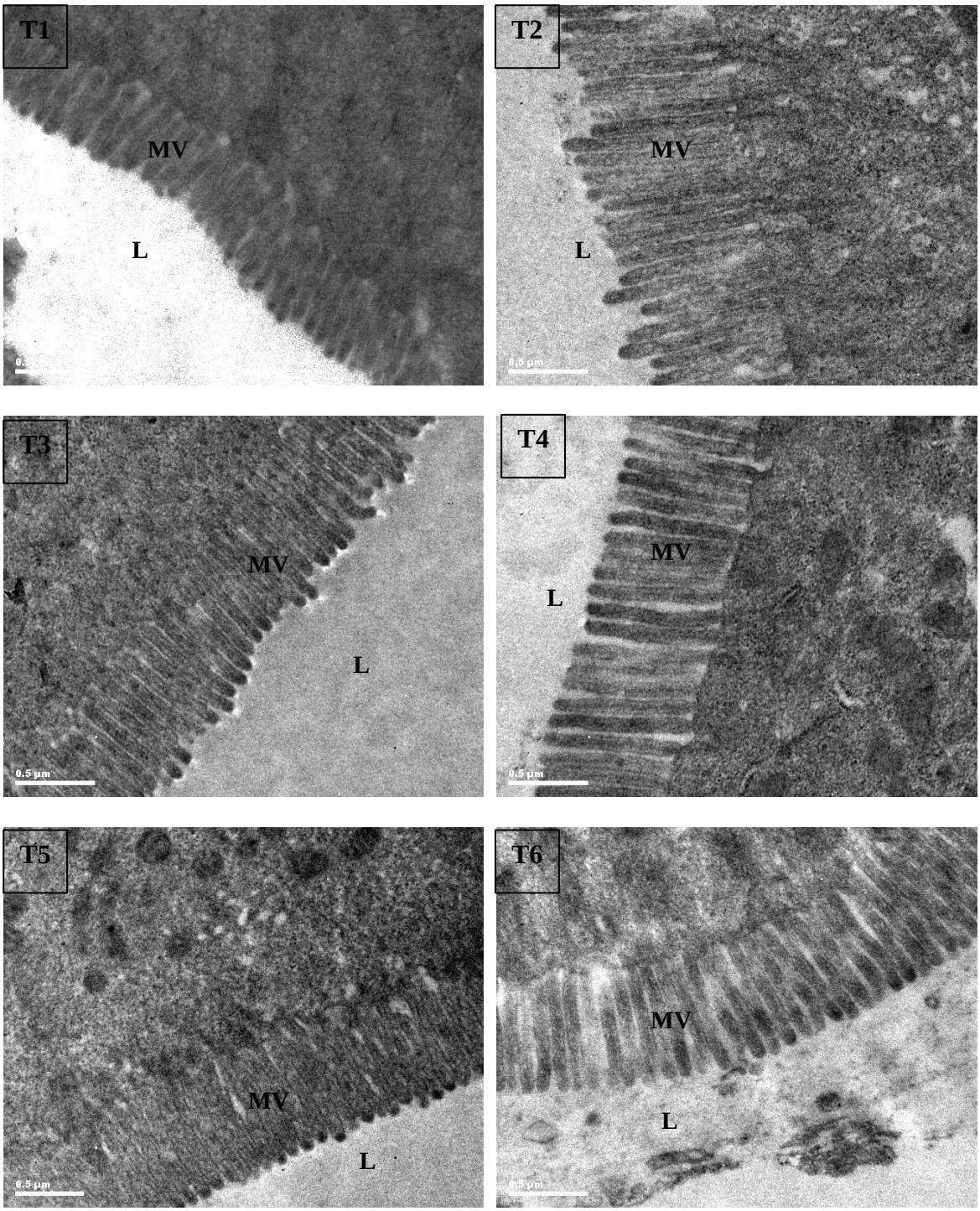


Figure 4.9 Transmission micrographs of microvilli of the mid-intestine of tilapia in different groups after feeding probiotic at 3 weeks (MV= microvilli; L= lumen; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=0.5 µm.

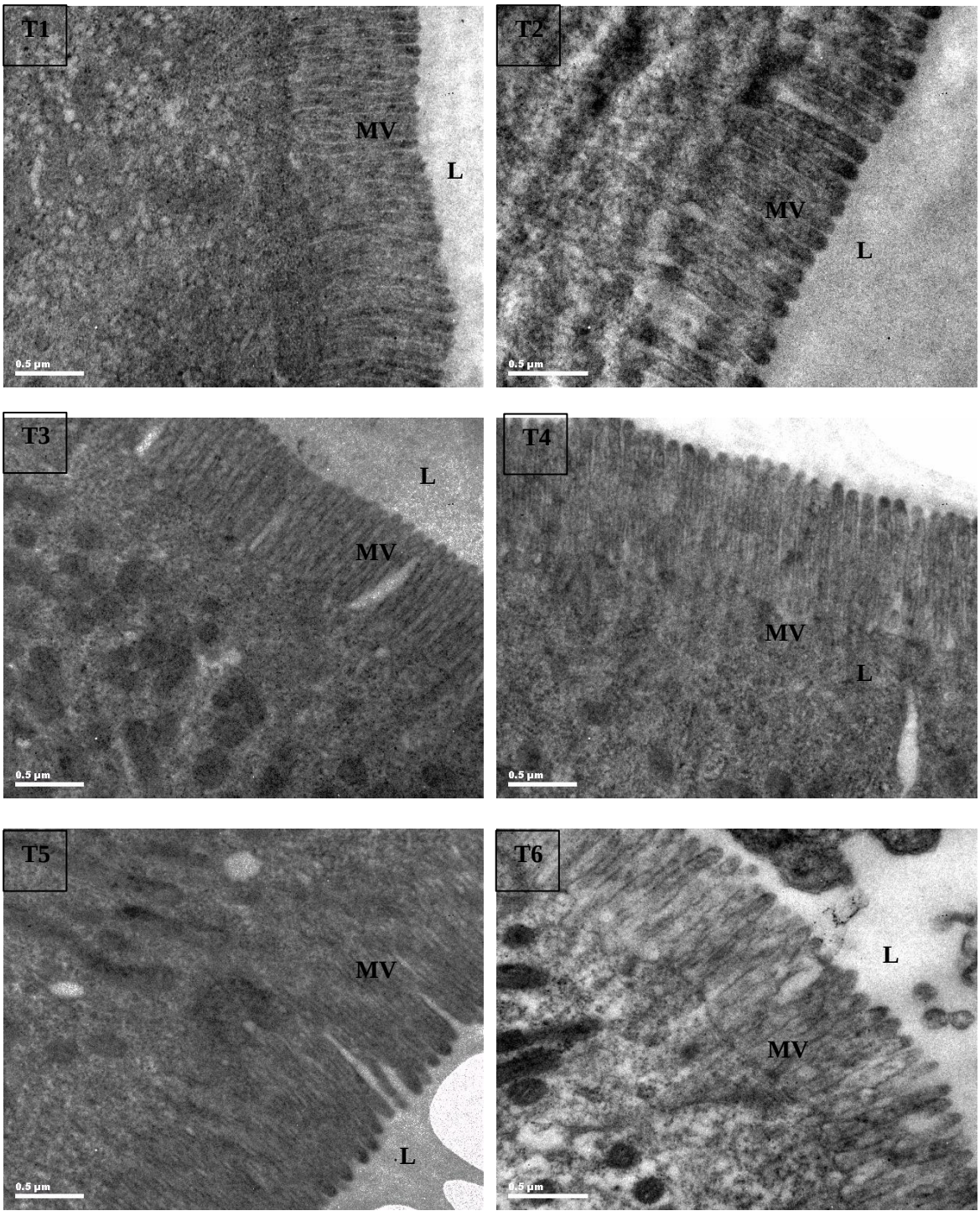


Figure 4.10 Transmission micrographs of microvilli of the mid-intestine of tilapia in different groups after feeding probiotic at 6 weeks (MV= microvilli; L= lumen; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=0.5 µm.

Table 4.5 Intestinal microvilli parameters of the tilapia of each treatment fed different probiotics at the trial mid point (week 3) and end point (week 6).

Treatments	Lenght (µm)		Width (µm)		Length/Width		Area (µm^2)	
	Week 3	Week 6	Week 3	Week 6	Week 3	Week 6	Week 3	Week 6
T1	0.578±0.040	1.080±0.036^{a}	0.095±0.012	0.077±0.010^{b}	6.212±0.849	14.370±2.083	0.180±0.029	0.265±0.037
T2	0.954±0.066	1.011±0.056^{a}	0.082±0.009	0.092±0.01^{a}	11.857±1.596	12.094±1.672	0.250±0.033	0.291±0.041
T3	0.684±0.047	0.752±0.039^{b}	0.077±0.009	0.080±0.010^{ab}	9.180±1.305	9.723±1.345	0.169±0.022	0.194±0.028
T4	0.831±0.036	0.900±0.083^{a}	0.096±0.008	0.093±0.013^{a}	8.809±0.967	9.809±1.597	0.256±0.023	0.274±0.051
T5	0.796±0.062	1.024±0.083^{a}	0.083±0.011	0.100±0.013^{a}	9.839±1.541	10.595±1.774	0.215±0.038	0.330±0.048
T6	0.774±0.030	0.954±0.056^{a}	0.086±0.008	0.085±0.010^{ab}	9.085±0.960	11.663±1.189	0.215±0.021	0.256±0.038

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column

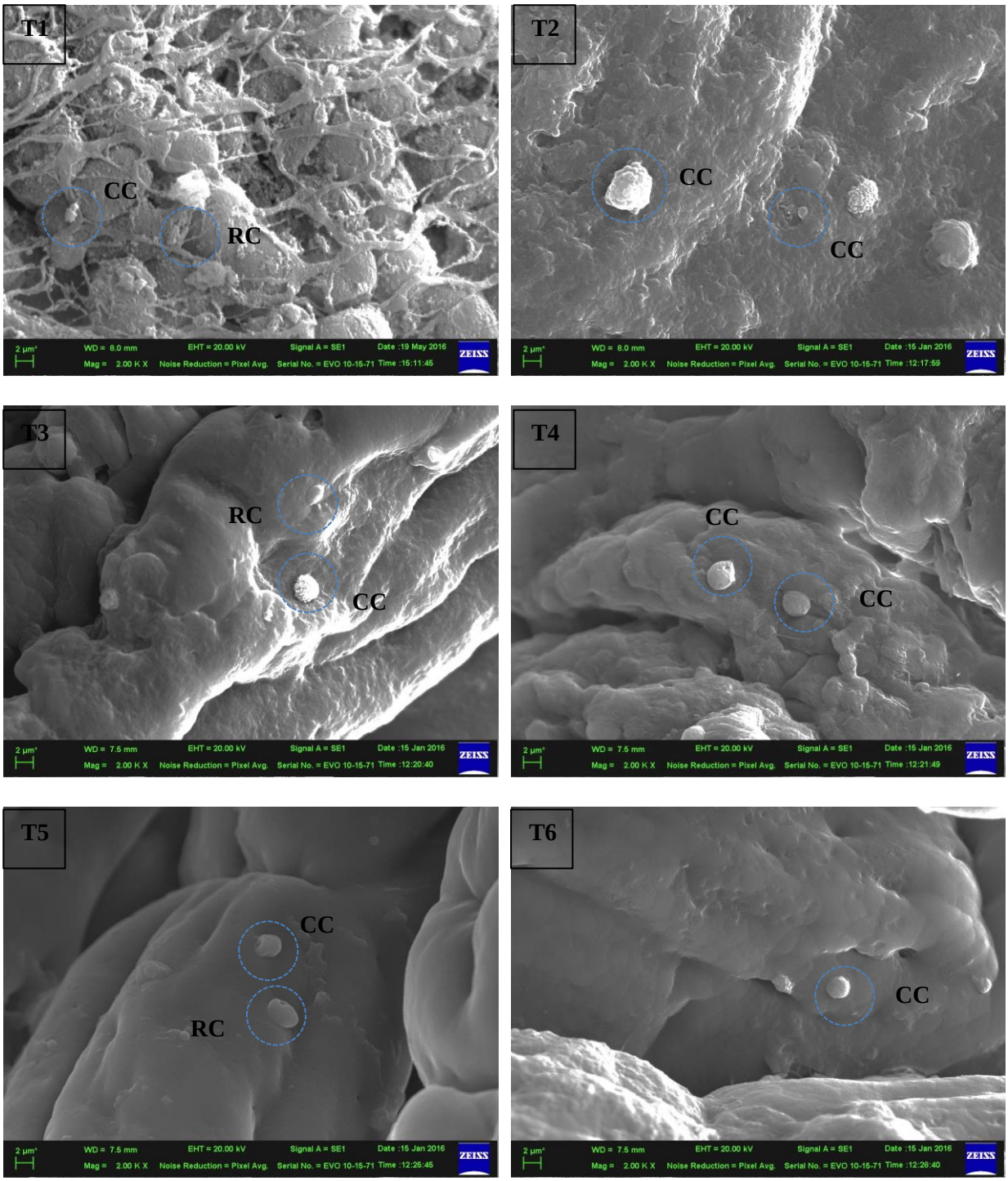


Figure 4.11 Scanning micrographs monitored bacterial colonization of the mid-intestine of tilapia in different groups after feeding probiotic at 3 weeks (CC=cocci-like-cell, RC=rod cell; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=2 μm.

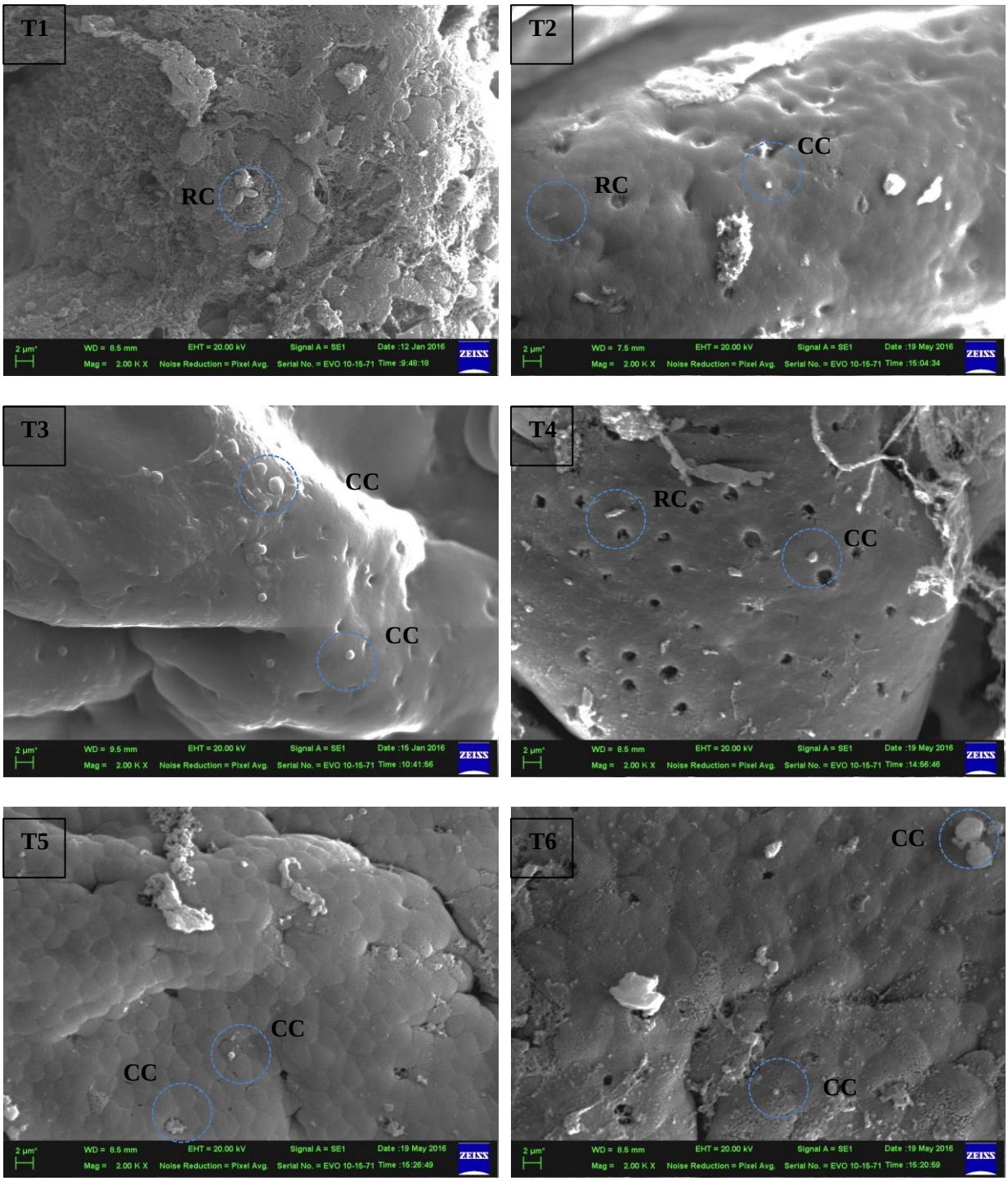


Figure 4.12 Scanning micrographs monitored bacterial colonization of the mid-intestine of tilapia in different groups after feeding probiotic at 6 weeks (CC=cocci-like-cell, RC=rod cell; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=2 μm.

4.4.4 Disease resistance

No mortalities were recorded with the mock challenge fish (sterile 0.85% NaCl injection). Fish mortalities in experimental diets were observed within 72 hours after injecting *A. hydrophila*. The *A. hydrophila* challenge led to mortality levels of 24±4% in the control fed fish. Probiotic feeding significantly (P<0.05) improved percent survival in all treatment groups (Figure 4.13), with levels of 96±4, 100, 98±4, 93±0 and 93±0 in T1, T2, T3, T4 and T5, respectively.

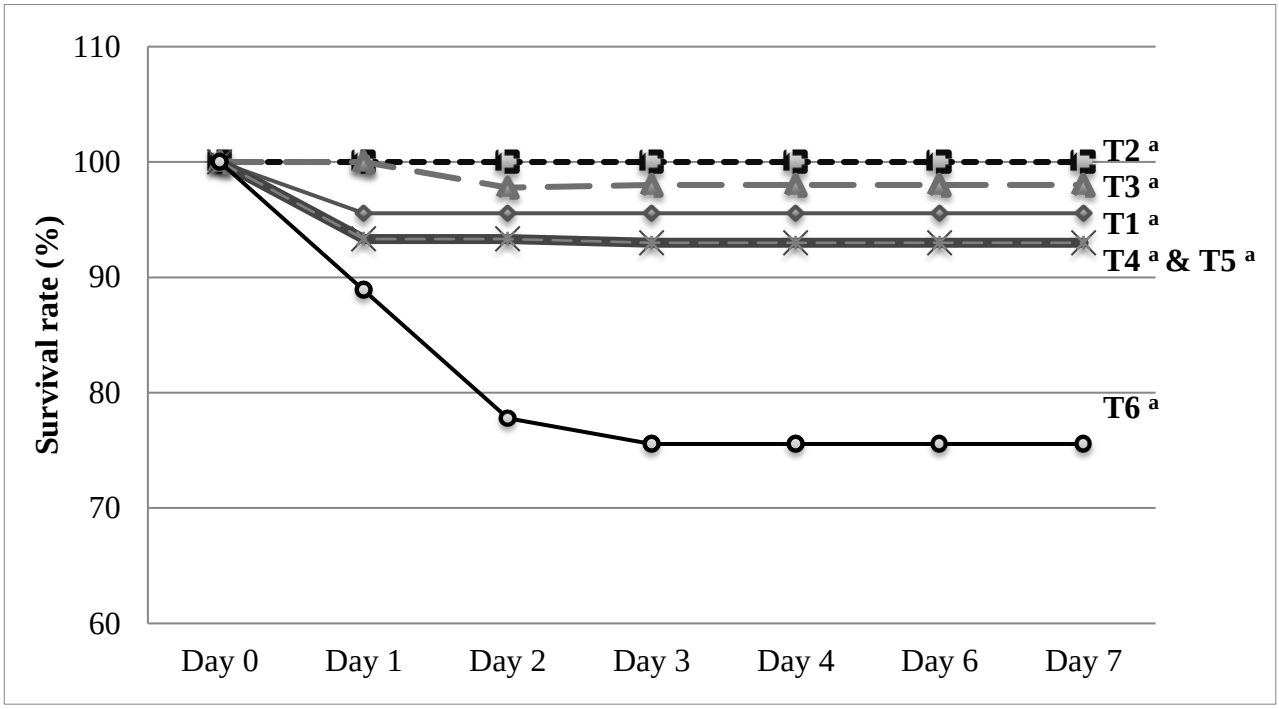


Figure 4.13 Survival rate of different groups after injecting pathogenic bacterium *A. hydrophila* for 7 days (T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group). Significant difference (P<0.05) between treatments denotes by different superscripts.

4.5 Discussion

After six weeks of the probiotic-feeding in this study, high growth performances, as evidenced by body weight, weight gain, average daily growth, and specific growth rate was observed in tilapia larvae fed autochthonous *Bacillus* spp. candidate probiotics (*Bacillus* spp. BRP01, BCHP02, and BRP00) and *Enterobacter* sp. NP02. Indeed, larvae fed these probiotics displayed the greatest weight gain, significantly greater than the control or *P. acidilactici* fed groups and the *Bacillus* sp. BRP01 fed larvae displayed significantly greater weight gain than all other treatments. Similar beneficial effects on growth performance been reported for tilapia larvae fed *Bacillus* based commercial probiotics (Aly *et al*., 2008; Nouh *et al*., 2009; He *et al*., 2013; Nakandakare *et al*., 2013). Apún-Molina *et al*. (2009) reported that an autochthonous *Bacillus* strain, administered directly through the feed and adding in rearing system could improve growth performances in tilapia larvae. In the present study, the commercial *P. acidilactici* investigated did not improve growth performance. Similarly, *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisae* combine have also failed to improve the growth performance of tilapia (Lara-Flores *et al*., 2003). According to this study, more benefits of *Bacillus* sp. RP01 supplemented in larval feed, which displayed to be 0.14±0.03 g of average daily growth and 0.07±0.03 g in the control group. Then, tilapia larvae fed with *Bacillus* RP01 can grow more than two folds without the probiotic-feeding, which might reduce financial farmers.

They were many evidences that fish feeding-probiotics might be affect high survival rates (84-96%) more than the control group (65 to 75%) and different survival rate might be found in different probiotic groups (Lara-Flores *et al*., 2003; Nouh *et al*., 2009). Non-effectiveness to survival rates of different probiotics comparing without probiotics have been reported that 70-85% of probiotic groups and 73% of the control groups (He *et al*., 2013). The highest survival rate both the probiotics and the control groups has distribute by Liu *et al*. (2013), who observed 93-100% in probiotic groups and 100% in the control group. Similar results of Nakandakare *et al*., (2013) reported

varying 97-100% both probiotics and the control group. In the present study, approximately 80% of the survival rate were not different between probiotic groups and the control group. Moreover, we found a few larvae dried causing by escape behavior to accompany with small hole of containers, however, treatments managed under the same conditions.

Probiotics may be associated with increased nutritional digestibility by producing several compounds to break down feed intake (El-Rhman *et al*., 2009). Then, high number of beneficial bacteria may reveal to high growth performances and this parameter is generally used to indicate the potential of probiotics. In this study, microbial loads showed no difference, both different media and group studies of two times. We found a few colonies of yeast, fungi and small colonies of bacteria occurring on MRS medium. Microbial loads (log cfu.g^{-1}) on TSA approximately found 7.15 to 8.94 and bacterial loads culturing by specific media were to be 6.17 to 8.02 of Gram-negative and 6.18 to 7.98 of *Bacillus* bacteria. He *et al*., (2013) reported both total allochthonous (approximately 8.48 to 8.88 log cfu.g^{-1}) and autochthonous bacteria (approximately 7.07 to 7.51 log cfu.g^{-1}) in the tilapia intestine no differed between the probiotic group (5×10^5 cells.g^{-1}) and the control group. Conversely, both allochthonous (approximately 5.09 to 5.41 log cfu.g^{-1}) and autochthonous bacillus (approximately 1.90 to 2.08 log cfu.g^{-1}) observed but non-occurrences in the control group. During the experiment at the midway and the end of the trial , *Bacillus* spp. probiotic candidates were detected both *Bacillus* spp. treatments and without receiving *Bacillus* spp. groups. The results indicated that *Bacillus* spp. corresponded colonization in tilapia larvae having the highest adhesive-potential of *Bacillus* sp. CHP02, which detected in triplicates of this treatment. *Bacillus* spp. might originate from the egg and the initial rearing water, which may allocate to the intestine. Several Bacilli are often reported to be diverse in freshwater ecosystem (Mohanty *et al*., 2011) and the GI tract of tilapia has been reported to identify several *Bacillus* strains (Al-Harbi and Uddin, 2004; Chantharasophon *et al*., 2011; He *et al*., 2013; Del'Duca *et al*., 2013). Moreover, these findings could support putative bacilli in the tilapia GIT and candidate probiotics of *Bacillus* strains display the adhesive-potential for colonization. All candidate probiotics were isolated a

single colony as wild type strain and then these colonies were sub-cultured to test the potential of probiotic properties *in vitro* trials for calculating the high potential of probiotic candidates (Chapter 3). Further studies, *Bacillus* spp. will be performed *in vitro* to select generation by generation of the adhesive-potential property as selected strain. Several bacterial morphologies colonized in the intestine indicating by SEM.

Probiotics may increase the absorptive surface index, microvilli length/density and/or goblet cell abundance in the intestinal tract of fish (Ferguson *et al*., 2010; Standen *et al*., 2015&2516; Adeoye *et al*., 2016; Handan *et al*., 2016). Therefore, histological studies using both light and electron microscopy were undertaken. Non-differences of goblet cells and microvilli parameters such as length, width, length/width proportion and area both at the midway and at the end of the trial between probiotic groups and the control group were found in this study. The results are not clear that probiotics may reveal positive effects goblet cells and microvilli characteristic but these parameters tend to increase following time studies. Some potential of bacilli probiotics on tilapia larvae have been reported to increase the thickness of the epithelial layer (Nakandakare *et al*., 2013). The SEM study can indicate several bacteria colonize in the intestine of tilapia larvae.

Fish feeding-probiotic may possibly be the effective prevention to pathogens. Variable disease resistances of fish fed probiotics have been demonstrated in several articles. For instance, tilapia fed with autochthonous probiotics *Pseudomonas* and *M. luteus* for 90 days and then these fish were used to inject pathogenic *A. hydrophila*. They showed different survival rates and only *M. luteus* increased survival rate (El-Rhman *et al*., 2009). The relative level of protection to *A. hydrophila* has been found in fish fed allochthonous Bacilli probiotic ($10^{12}g^{-1}$) for 30 days more than the control group (Aly *et al*., 2008). According to Villamil *et al*., (2014) used allochthonous probiotic *Lac. acidophilus* (10^6 cfu.g^{-1} diet) fed tilapia for 15 days. These fish were infected with pathogenic *A. hydrophila* and displayed 80% of the survival rate more than the control group (50%). Similar result was found in this study, tilapia larvae fed probiotics displaying high survival rates (96%) than the

control group (76%). Conversely, probiotics have been used to feed fish for 56 days, but fish not succeeded to resist pathogens (Nouh *et al*., 2009).

In conclusion, three strains of autochthonous *Bacillus* displayed high potential as probiotics in this *in vivo* larval evaluation. The greatest potential was observed with *Bacillus* sp. RP01, which supported the highest average body weight, total weight gain, average daily growth and specific growth rate after feeding for three weeks. Chapter 5 will evaluate the effect of these probiotic candidates on growth performances of tilapia in the later stages in the growing cycle.

Chapter 5

In vivo trial using tilapia juvenile

5.1 Abstract

Male tilapia were fed with one of six different commercial diets containing potential probiotics at 10^{6-7} cfu.g^{-1}: T1, T2, T3, T4, T5 or T6. Two thousand five hundred of tilapia (6.96±1.74 g) into triplicate cement ponds (600L of capacity). Samples were reared in the cement ponds (2.5L.min^{-1} of flow rate) for 10 weeks. At the end of the trial, no significant differences between the treatment groups (P>0.05) of body weight, increasing weight, total length, increasing length, specific growth rate, K factors, RIL, feed conversion ratio and survival rate were observed. The levels of cultivable microbes in the intestine (log cfu.g^{-1}), the abundance of intestinal goblet cells and microvilli length displayed no significant differences between the treatment groups (P>0.05). Significant differences between the treatment groups (P<0.05) were observed for microvilli width, the proportion of microvilli length/width and microvilli area. Significant differences (P<0.05) of glucose and plasma osmolality between groups were found in stressed fish to induce by pathogenic *A. hydrophila*, and not different for plasma cortisol. The highest level of plasma glucose was found in T3 and the lower in T2. Plasma osmolality was found the highest in T1 and the lowest in T2. Fish induced stress by using thermal condition, significant differences among groups (P<0.05) were found in plasma cortisol and osmolality and plasma glucose displayed no difference. The highest level of cortisol was found in T3 and the lowest was found in T4. The highest plasma osmolality was observed in T6 and lowest in T1. Fish fed different diets were observed low survival rates after injecting pathogen

and showed no difference ($P<0.05$) between treatment groups. The thermal induction was displayed 100% of survival rates in all treatments ($P<0.05$).

5.2 Introduction

Tilapia culture is experiencing great growth annually. In 2030, tilapia production is predicted to increase to 7.3 million metric tonnes from 4.3 million metric tonnes in 2010 (The World Bank, 2013). As a versatile species, tilapia are cultured in many systems such as the earthen pond, cages in ponds, plastic tanks, cement ponds and cages in lakes. Generally, high productivity in each crop production is a target of farmers, who usually rear at high density with high feed inputs. These high stocking densities and high load pollutions can cause poor water qualities and induce stress, which can lead to the spread of disease mortalities. Traditionally, veterinary medicines, chemicals, antibiotics, parasiticides, feed additives and probiotics are used to achieve healthy fish and to prevent or treat disease outbreaks (Rico *et al*., 2013). A recent study reported that 84% of probiotics use in aquatic farms in Asia is used to as an attempt to improve water quality and reduce stress conditions and 16% for mixing in feeds (Rico *et al*., 2013).

The previous studies, we selected autochthonous probiotic candidates (Chapter 3) as *Bacillus* sp. CHP02, RP01 & RP00 and *Enterobactor* sp. NP02 evaluated in tilapia fry (Chapter 4) by comparing with a commercial probiotic *P. acidilactici* as a reference strain and the control group (without probiotic in fish feed). The high effective of probiotic candidates was found in *Bacillus* sp. RP01, which revealed to high average body weight, total weight gain, average daily growth, and specific growth rate. *Bacillus* sp. RP01 can display colonization in the intestine of tilapia larvae after feeding for three weeks. Then, the aims of this study were to evaluate these probiotic candidates in grow-out tilapia, which observed growth performances and microscopic studies (LM, TEM and SEM) for evaluating the histological changes of intestine, microvilli and bacterial

colonization, in addition, fish samples after the post probiotic-feeding were induced stresses by using pathogenic injection and thermal shock to evaluate physiological responses.

5.3 Materials and methods

5.3.1 Nile tilapia preparation

Two thousand five hundred sex reversed male tilapia larvae at the age of 50dph were transferred from AIT to KMITL within an hour. These fish were reared in cement ponds with aeration and flow-through water (8 $l.min^{-1}$ of flow rate). After acclimating for a week, 800 fish having body weight of 3-4 g received a microchip (8 mm long × 1 mm diameter, low−frequency around 134.2 kHz which refer to ISO11784/11785 animal ID transponder FDX−B) injected into the ventral cavity. These fish were acclimated for 3 weeks in cement ponds to allow for recovery and repairing tissue damage which may have resulted from tag implantation (Meeanan *et al*., 2009). During the acclimation period they were fed with twice basal fish feed per day (Premafeed Co., Ltd.: 1.2 mm of diameter, 12% of moisture, 30% of crude protein, 3% of total fat, and 12%).

5.3.2 Experimental trial

Six different commercial diets containing potential probiotics at 10^{6-7} $cfu.g^{-1}$: T1: (*Bacillus* sp. CHP02), T2: (*Bacillus* sp. RP01), T3: (*Bacillus* sp. RP00), T4: (*Enterobacter* sp. NP02), T5: (*P. acidilactici*) or T6: (control group – no probiotic) were performed with three replicates in this study. A total of 726 tagged fish (6.96±1.74 g of average weights) were distributed into eighteen ponds (about 40 tagged fish per pond) having 600L of pond capacity and flow rate of 2.5 $l.min^{-1}$. Then, all ponds were added residual fish adjusting 395.70 to 456.65 g of the total weight ($P<0.05$). These ponds used plastic nets to cover for fish rearing to support reduced time handle (Figure 5.1). Probiotics and fish feed were prepared as described in section **2.5**. Fish fed three times per day at the rate of 10% biomass in the first week, 6% biomass in the second to the third weeks and then 4% biomass was used to feed fish to the end of the trial. Fish were starved 24 hours and then fish

weight in each pond was recorded. Fish dead was monitored and removed daily, while fish excreta were drained twice per week.

Figure 5.1 Fish rearing management at KMITL. A: 600L of the cement ponds use flow through system, B: the plastic nets use to support fish handling, and C: Daily fish feed of each pond is separately kept in each container.

During the experimental period, water quality parameters were measured weekly, which found to be 30.6±0.3^{0}C of water temperature, 4.70±0.35 mg.l^{-1} of dissolved oxygen (DO), 6.8±0.2 of pH and 0.41±0.05 mg−N.l^{-1} of total ammonia.

5.3.3 Growth performances

After a 24-h of feed deprivation period, the morphometric of tagged fish were recorded each week as described in section **2.5** by using Retina System (Matcha IT, Thailand). Individual quantitative

data were used to weekly analyze parameters of growth performances in terms of average body weight, increasing weight, WG, average total length, increasing length, TLG, ADG and K factor. At the trial mid point and the trial ending, parameters of the RIL and FCR were determined. These were calculated equations as described in **2.5.1**. The SR (%) at the end of the trial was calculated (described as **2.5.2**).

5.3.4 Bacterial studies

5.3.4.1 Plating and colony counts

Intestinal cultivable microbial loads were determined at the midway (5 weeks) and at the end of the trial (10 weeks). Fish were deprived of feed for 24 hours and then three fish from each pond were dissected to remove the GIT (as described in **2.2**). An individual sample (Figure 2.1: part 4) was used serial dilution to estimate a viable count by spreading method (as explained in **2.3.1**). A volume of 100 µL of 10^{-1}, $10^{-3\text{-}4}$, $10^{-3\text{-}4}$ and $10^{-7\text{-}8}$ serial dilutions was spread onto duplicate MRS-A, EMB (Himedia, India), BA medium and TSA, respectively. Agar plates were incubated at 32 ^{0}C for 48 hours and then recorded photographs of colony-forming units (cfu.g^{-1}) of plates. The ImageJ 1.48v software (national Institutes of Health, USA) was used to count for microbial colonies.

5.3.4.2 Probiotic monitoring

Triplicate intestines from each replicate (section ***5.3.4.1***) used (Figure 2.1: part 3) to extract genomic DNA by using a Qiagen DNA extraction kit (section ***2.3.5.1***). In brief, triplicated intestine in each replicate (Figure 2.1: part 3) were homogenized in ASL buffer and then samples were centrifuged to remove supernatant to mix with Inhibit EX tablet. Samples were centrifuged to remove supernatant and then added Proteinase K and AL buffer in samples. Samples were incubation and added absolute ethanol. Finally, samples were washed with AW1 and AW2 buffers. Genomic bacteria were maintained in AE buffer. These genomic samples used to monitor the presence of probiotic *Bacillus* spp., *Enterobactor* sp. and *P. acidilactici* in the GIT by using specific

probiotic primers (Table 2.1). The genomic DNA of each replicate was pooled into a single sample. The total volume of PCR was 25 µl: 12.5 µl of the GoTaq® Green Master Mix, 2.5 µl of 10 µM of each primer, 1 µl of DNA template and 6.5 µl of sterile distilled water. The cycling conditions were dependent on the different probiotic primers, as explained in ***2.3.5.2***. The PCR products were checked using agarose gels 1.5% (w/v) containing RedSafe DNA Stain (0.005 %) as explained in ***2.3.5.3***. Document gels were interpreted comparing with positive probiotic bands created from known probiotic isolates.

5.3.5 Microscopic studies

At the trial mid point and the end of the trial, samples (section ***5.3.4.1***) used the mid-intestinal tract (Figure 2.1: part 1) for studying the intestinal histology by using LM. Samples were prepared as described in ***2.5.3.1***. These samples were analyzed to determine the density of goblet cells (cell/0.1mm^2) by using the ImageJ 1.48v software (National Institutes of Health, USA).

At the mid-trial and the trial ending, the mid-intestine of triplicate samples of each treatment (Figure 2.1: part 2) was prepared as explained in ***2.5.3.2***. Samples were randomized to estimate both length and width microvilli by using TEM (Phillips: Techni20, Holland). The ImageJ 1.48v software (national Institutes of Health, USA) was used to measure microvilli length (h_{mi}) and microvilli width (w_{mi}) from the micrographs. Microvilli areas of samples were calculated by using the equation of $2\pi rh+\pi r^2$ (r=radian of microvilli; $w_{mi}/2$, and h=microvilli length; h_{mi} as Figure 2.5).

Microbial colonization in the intestine of tilapia at the trial mid point and the end of the trial was observed by using a SEM studies. Triplicate samples of the mid-intestine (Figure 2.1: part 2) of each treatment were prepared as described in ***2.5.3.3***. These samples were dehydrated and coated gold (Cressington Sputter Coater, 108 auto). Samples were scanned and imaged to assess the microbial colonization on the intestinal epithelial cells using a SEM (Carl Zeiss: EVO® HD, USA).

5.3.6 Stress inductions

At the end of the feeding trial (70 days), fish samples were separated into two groups for stressed challenges: 1] pathogenic, and 2] thermal shock (Figure 5.2). For the disease challenge, thirty fish of each container were IP injected with 0.5 ml of *A. hydrophila* suspended in sterile 0.85% NaCl (1×10^{10} cfu.ml^{-1}). Duplicate sets of fish (n=30) were IP injected with 0.5 ml of sterile 0.85% NaCl as the negative control group. For the thermal challenge: ten individual fish from each container were exposed to 40^{o}C water for 30 minutes. These stressed fish were maintained separately in ponds for 7 days to monitor fish mortality.

Triplicated samples of stressed fish in each pond were randomly selected to take blood samples for measuring osmolality, glucose and cortisol. A total of 1ml of blood sample was obtained from the caudal vein by using a heparinized syringe. The time of this process was less than 1 minute. Blood samples were taken to centrifuge at 1000 g for 10 min and plasma samples were collected and stored at − 20˚C for further studies.

Cortisol levels were measured using a cortisol ELISA kit (Cayman Chemical, USA) following the manufacturer's instructions. A total of 500 µl plasma volume was added with tritium-labeled cortisol and then adjusted pH to 2 by using 5 M HCl. Samples were extracted by using methylene chlorine and heated at 30^{o}C under a gentle stream of nitrogen. Samples were extracted in 0.5 ml of ELISA buffer. These samples and cortisol standard were measured the optical density at 420 nm by using Micro-plate Reader (Sunrize, Tecan Austria GmbH).

Plasma glucose was measured by using Dinitrosalicylic colorimetric method (DNS method) according to Miller (1959) with some modification. A total volume of 100 ml DNS reagent (1 g of dinitrosalycyclic acid: DNS, 1 g of NaOH, 20 g of NaK tartrate (Rochelle salt), 0.05 g of sodium sulfite and 0.2 ml of Phenol; melted at 60 ^{0}C) was prepared and kept in a dark bottle. A volume of 25 µl plasma was mixed with 225 µl of distilled water and 250 µl of DNS and then homogenized

using vortex mixer. Duplicate negative controls were prepared without plasma sample. Samples were heated at 100^{o}C for 3 minutes. Then 50 µl of cool sample was added into duplicate wells of 96-well microl plate and 50 µl of distilled water was added into duplicate wells as a negative control. The absorbance of samples was read with a Micro-plate Reader (Sunrize, Tecan Austria GmbH) at a wavelength of 570 nm. A standard glucose curve was constructed by using different concentrations of glucose between 0.000 to 0.600 µl.l^{-1} having 0.0465 to 1.0470 of absorbance volumes, which formulated the following equation: $y = -0.0871 + 1.7509X$ (R^2=0.983). The estimation of glucose concentrations (mg.ml^{-1}) was calculated by using this standard curve.

A total volume of 50 µl of plasma of each sample was transferred into microfuge tube having 200 µL of sizing to measure the plasma osmolality by using the Gonotec machine (Osmomat 030).

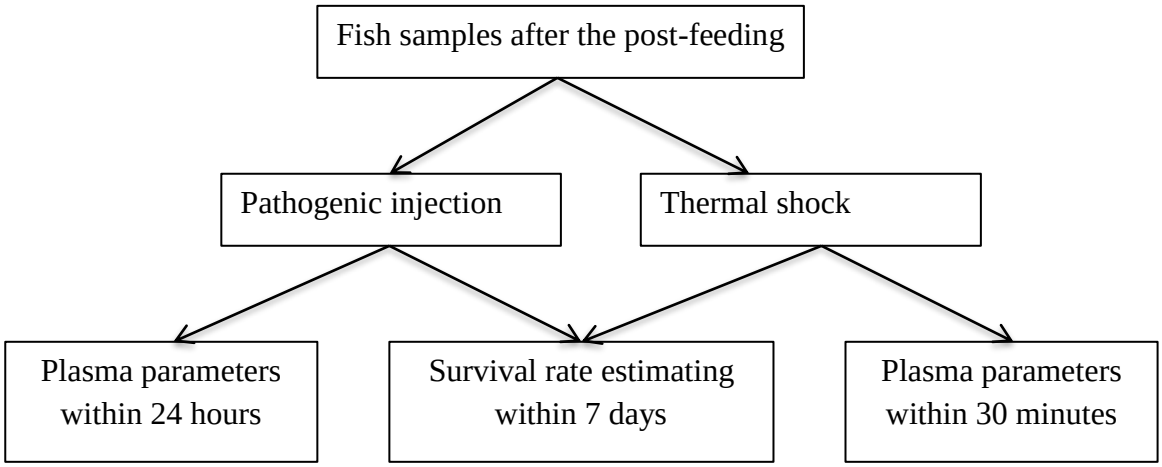


Figure 5.2 Flow diagrammatic stress inductions in samples after the ending of the trial feeding.

5.3.7 Statistical analysis

The results were presented as means and standard deviation. The percentage and viable counts data were transformed to ensure normality. Growth performances, log viable counts, blood parameter and other parameters were compered using a one-way analysis of variance (ANOVA). Significant differences between groups were accepted at $P < 0.05$. Pairwise comparison probabilities were used

to compere different among means of treatments. Analyses were carried out statistical data by using the Systat software ver. 5.02 (Illinois, USA).

5.4 Results

5.4.1 Growth performances

The growth performances of body weight during 10 weeks of treatments was presented in Table 5.1, increasing weight in Table 5.2, total lengths in Table 5.3, increasing lengths in Table 5.4, SGR in Table 5.5, ADG in Table 5.6 and K factors in Table 5.7. Significant differences ($P<0.05$) between treatments for growth performances were observed in average body weights of 8 weeks, average of increasing weights at 1 week, average of increasing lengths at 1 week, specific growth rates at 1 and 2 weeks, average daily growth at 1 week and K factors at 1, 2 and 3 weeks. The total weights of the experimental diets were observed in Table 5.8. However, these parameters were no longer significantly different at the end of the trial. The RIL (Figure 5.3) and FCR (Figure 5.4) were not significantly different ($P>0.05$) between treatment groups.

Table 5.1 Average body weights (g) of different treatments in each week.

Treatments	The initial mean of wet weight	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	7.00±2.22 (n=120)	9.62±3.12 (n=120)	12.56±4.19 (n=118)	16.28±5.56 (n=118)	18.88±6.50 (n=116)	22.03±7.44 (n=112)	26.38±8.99 (n=111)	31.47±10.49 (n=105)	37.69±12.50^{a} (n=103)	47.67±16.59 (n=102)	54.14±18.95 (n=101)
T2	6.97±2.19 (n=120)	9.61±3.41 (n=119)	12.78±4.48 (n=114)	16.25±5.99 (n=110)	19.32±7.15 (n=107)	22.24±7.76 (n=103)	27.05±8.62 (n=98)	31.76±9.96 (n=93)	37.26±11.70^{a} (n=90)	48.96±14.85 (n=92)	53.81±16.69 (n=89)
T3	6.93±2.48 (n=120)	9.54±3.62 (n=119)	12.84±4.84 (n=118)	16.18±6.13 (n=114)	19.26±7.31 (n=107)	22.56±8.49 (n=101)	27.26±9.99 (n=101)	31.74±10.83 (n=98)	37.46±12.55^{a} (n=97)	48.25±16.39 (n=93)	53.92±18.15 (n=96)
T4	6.86±2.13 (n=123)	10.13±3.18 (n=120)	13.38±4.15 (n=116)	16.75±5.38 (n=116)	19.35±6.25 (n=116)	22.70±7.08 (n=116)	27.12±8.17 (n=108)	31.70±9.34 (n=107)	37.32±10.84^{a} (n=106)	49.32±14.49 (n=103)	55.42±16.03 (n=103)
T5	6.91±1.87 (n=123)	9.97±2.86 (n=121)	12.73±3.75 (n=119)	15.80±5.08 (n=116)	18.92±5.87 (n=115)	21.55±6.56 (n=114)	25.32±7.90 (n=108)	29.33±9.04 (n=106)	34.65±10.52^{bc} (n=101)	44.51±14.06 (n=101)	50.82±15.45 (n=101)
T6	7.11±2.04 (n=120)	10.27±3.23 (n=119)	13.09±4.47 (n=115)	16.52±5.72 (n=115)	19.30±6.74 (n=111)	22.39±7.76 (n=109)	26.34±9.30 (n=104)	30.41±10.72 (n=103)	36.87±12.43^{ab} (n=95)	47.33±16.16 (n=96)	53.31±18.25 (n=93)

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column. The number of tagged fish in parenthesis is denoted n values.

Table 5.2 Average of increasing weights (g) of different treatments in each week.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	2.62±1.18^{b}	5.53±2.37	9.16±3.91	11.85±5.03	14.96±6.13	19.27±7.80	24.25±9.49	30.44±11.57	40.43±15.76	46.87±18.24
T2	2.63±1.40^{bc}	5.77±2.59	9.30±4.19	12.26±5.48	15.14±6.19	19.83±7.16	24.45±8.59	30.00±10.34	41.71±13.65	46.55±15.39
T3	2.64±1.36^{bcd}	5.91±2.65	9.30±4.13	12.32±5.39	15.54±6.56	20.27±8.11	24.53±9.20	30.24±10.83	41.06±14.70	46.74±16.59
T4	3.28±1.34^{a}	6.44±2.46	9.81±3.79	12.40±4.76	15.76±5.68	20.09±6.93	24.65±8.10	30.30±9.65	42.37±13.41	48.36±14.92
T5	3.07±1.20^{a}	5.81±2.28	8.86±3.79	11.99±4.69	14.63±5.52	18.39±6.96	22.48±8.35	27.69±9.75	37.56±13.32	43.86±14.75
T6	3.13±1.34^{a}	5.93±2.78	9.38±4.12	12.14±5.24	15.17±6.37	19.14±7.95	23.25±9.38	29.67±11.10	40.09±14.83	46.21±16.98

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.3 Average total lengths (cm) of different treatments in each week.

Treatments	The initial mean of length	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	7.45±0.75	8.18±0.88	8.82±0.99	9.53±1.10	10.09±1.22	10.63±1.25	11.16±1.35	11.78±1.45	12.49±1.51	13.26±1.68	13.96±1.76
T2	7.42±0.77	8.10±0.97	8.79±1.10	9.45±1.29	10.09±1.40	10.70±1.41	11.29±1.34	12.01±1.70	12.54±1.43	13.42±1.48	14.00±1.58
T3	7.46±0.85	8.11±1.02	8.80±1.15	9.45±1.25	10.12±1.36	10.72±1.46	11.22±1.53	12.90±1.51	12.52±1.58	13.26±1.73	13.83±1.79
T4	7.53±0.67	8.26±0.83	8.95±0.92	9.66±1.04	10.26±1.12	10.78±1.18	11.32±1.20	11.95±1.27	12.60±1.34	13.48±1.50	14.04±1.53
T5	7.50±0.64	8.18±0.74	8.87±0.86	9.53±0.99	10.13±1.06	10.64±1.12	11.15±1.21	11.63±1.25	12.25±1.34	13.13±1.49	13.77±1.48
T6	7.48±0.74	8.22±0.92	8.93±1.10	9.56±1.24	10.20±1.35	10.71±1.45	11.25±1.58	11.79±1.66	12.43±1.74	13.22±1.88	13.84±2.04

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.4 Average of increasing lengths (cm) of different treatments in each week.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	$0.73±0.23^{b}$	1.36±0.39	2.06±0.57	2.62±0.73	3.16±0.83	3.67±0.97	4.27±1.13	4.97±1.22	5.73±1.41	6.44±1.50
T2	$0.68±0.25^{bc}$	1.35±0.44	2.00±0.65	2.63±0.80	3.21±0.86	3.75±0.85	4.45±1.26	4.99±0.99	5.87±1.09	6.45±1.19
T3	$0.66±0.22^{bcd}$	1.33±0.42	1.98±0.59	2.63±0.74	3.21±0.85	3.71±0.95	4.34±0.97	4.97±1.05	5.71±1.22	6.28±1.30
T4	$0.73±0.23^{a}$	1.40±0.40	2.11±0.55	2.71±0.65	3.23±0.76	3.74±0.83	4.37±0.91	5.02±0.97	5.93±1.16	6.46±1.19
T5	$0.70±0.20^{a}$	1.37±0.39	2.03±0.57	2.64±0.68	3.14±0.79	3.64±0.91	4.13±0.99	4.75±1.09	5.63±1.28	6.28±1.27
T6	$0.73±0.25^{a}$	1.42±0.49	2.08±0.67	2.69±0.81	3.20±0.94	3.73±1.08	4.29±1.19	4.91±1.26	5.69±1.41	6.36±1.56

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.5 Specific growth rates of individual fish tagged in different treatments.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	4.42±1.49^{bc}	4.04±1.06^{c}	3.85±1.00	3.44±0.88	3.18±0.76	3.06±0.70	2.95±0.66	2.91±0.59	2.94±0.57	2.83±0.54
T2	4.23±2.06^{c}	4.14±1.16^{abc}	3.86±1.01	3.45±0.91	3.16±0.73	3.10±0.56	2.97±0.49	2.89±0.46	3.01±0.46	2.84±0.40
T3	4.41±1.79^{bc}	4.26±1.14^{abc}	3.92±1.04	3.51±0.89	3.23±0.76	3.15±0.68	2.99±0.57	2.92±0.51	2.99±0.49	2.85±0.48
T4	5.56±1.07^{a}	4.64±1.03^{a}	4.13±0.89	3.61±0.78	3.35±0.66	3.18±0.59	3.05±0.52	2.96±0.48	3.09±0.45	2.92±0.42
T5	5.16±1.26^{ab}	4.28±1.05^{abc}	3.81±1.04	3.52±0.85	3.20±0.74	3.04±0.68	2.90±0.63	2.83±0.57	2.91±0.56	2.81±0.48
T6	5.02±1.23^{ab}	4.09±1.25^{bc}	3.82±1.05	3.40±0.90	3.12±0.81	2.97±0.76	2.84±0.70	2.82±0.60	2.90±0.54	2.80±0.53

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.6 Average daily growths of individual fish tagged in different treatments.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	0.37±0.17^{c}	0.40±0.17	0.44±0.19	0.42±0.18	0.43±0.18	0.46±0.19	0.50±0.19	0.54±0.21	0.64±0.25	0.67±0.26
T2	0.38±0.20^{bc}	0.41±0.19	0.44±0.20	0.44±0.20	0.43±0.18	0.47±0.17	0.50±0.18	0.54±0.18	0.66±0.22	0.67±0.22
T3	0.38±0.19^{bc}	0.42±0.19	0.44±0.20	0.44±0.19	0.44±0.19	0.48±0.19	0.50±0.19	0.54±0.19	0.65±0.23	0.67±0.24
T4	0.47±0.19^{a}	0.46±0.18	0.47±0.18	0.44±0.17	0.45±0.16	0.48±0.17	0.50±0.17	0.54±0.17	0.67±0.21	0.69±0.21
T5	0.44±0.17^{abc}	0.42±0.16	0.42±0.18	0.43±0.17	0.42±0.16	0.44±0.17	0.46±0.17	0.49±0.17	0.60±0.21	0.63±0.21
T6	0.45±0.19^{a}	0.42±0.20	0.45±0.20	0.43±0.19	0.43±0.18	0.46±0.19	0.47±0.19	0.53±0.20	0.64±0.24	0.66±0.24

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.7 K factors of individual fish tagged in different treatments.

Treatments	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	1.70 ± 0.12^{b}	1.76 ± 0.10^{b}	1.79 ± 0.13^{abc}	1.76 ± 0.13	1.75 ± 0.11	1.81 ± 0.13^{a}	1.84 ± 0.13^{ab}	1.85 ± 0.12	1.95 ± 0.20	1.90 ± 0.23
T2	1.71 ± 0.15^{b}	1.79 ± 0.12^{ab}	1.83 ± 0.13^{a}	1.77 ± 0.13	1.73 ± 0.13	1.80 ± 0.10^{abc}	1.78 ± 0.16^{abc}	1.81 ± 0.11	1.95 ± 0.11	1.90 ± 0.28
T3	1.70 ± 0.14^{b}	1.79 ± 0.11^{ab}	1.82 ± 0.12^{ab}	1.76 ± 0.13	1.73 ± 0.11	1.83 ± 0.13^{a}	1.80 ± 0.11^{ab}	1.82 ± 0.11	1.97 ± 0.13	1.95 ± 0.22
T4	1.74 ± 0.11^{ab}	1.81 ± 0.10^{a}	1.79 ± 0.11^{abc}	1.72 ± 0.10	1.75 ± 0.10	1.81 ± 0.11^{ab}	1.80 ± 0.11^{ab}	1.80 ± 0.11	1.95 ± 0.15	1.94 ± 0.21
T5	1.77 ± 0.11^{a}	1.77 ± 0.10^{ab}	1.75 ± 0.12^{c}	1.75 ± 0.10	1.73 ± 0.09	1.76 ± 0.11^{bc}	1.79 ± 0.10^{ab}	1.82 ± 0.14	1.89 ± 0.10	1.88 ± 0.18
T6	1.78 ± 0.10^{a}	1.75 ± 0.11^{b}	1.79 ± 0.10^{abc}	1.73 ± 0.10	1.72 ± 0.11	1.75 ± 0.12^{c}	1.75 ± 0.11^{c}	1.82 ± 0.11	1.95 ± 0.20	1.90 ± 0.12

Presented values are means of triplicates ± standard error of mean and denoted significant differences ($P<0.05$) between treatments in each week by using different superscripts in each column.

Table 5.8 Total weights (g) of each treatment in each week during the experimental diets.

Treatments	The initial weight	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10
T1	1337	1848	2404	2404	3520	4026	4438	5010	5915	7404	8280
T2	1336	1808	2364	2364	3424	3842	4182	4635	5480	7101	8049
T3	1335	1784	2377	2377	3306	3652	3941	4449	5177	6684	7482
T4	1339	1802	2322	2363	3326	3926	4173	4728	5556	7315	8239
T5	1339	1860	2366	2366	3443	3889	3967	4563	5182	6426	7402
T6	1346	1868	2310	2321	3355	3873	4235	4730	5624	7001	8004

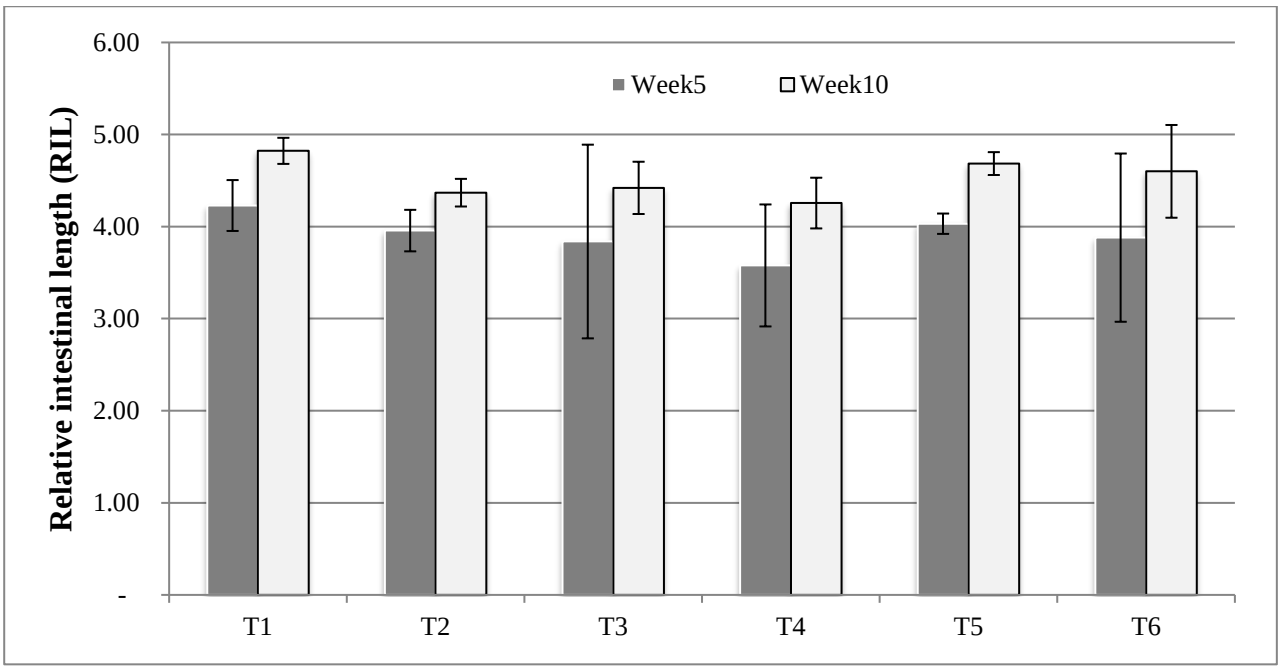


Figure 5.3 RIL of different treatments at the mid-trial (5 weeks) and the end of the trial (10 weeks) of experimental feeding. Presented values are means of triplicates ± standard error of mean.

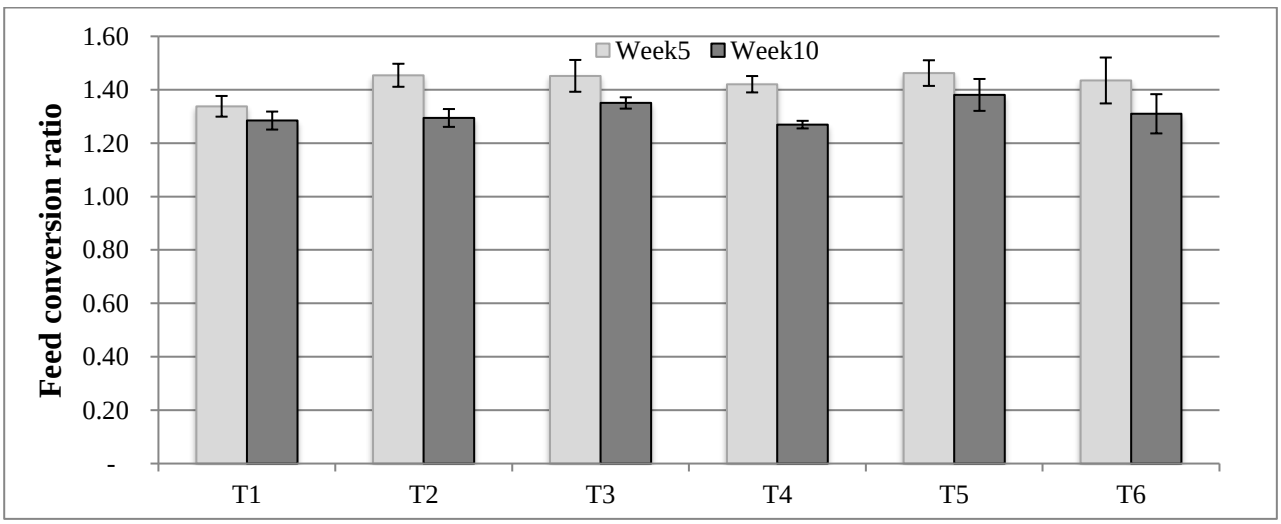


Figure 5.4 FCR of samples fed different diets at the mid-trial (5 weeks) and the end of the trial (10 weeks). Presented values are means of triplicates ± standard error of mean.

The survival ranged from 79 to 83%. There were no significant differences in survival rates between treatment studies (Figure 5.4) at the (P>0.05), which displayed

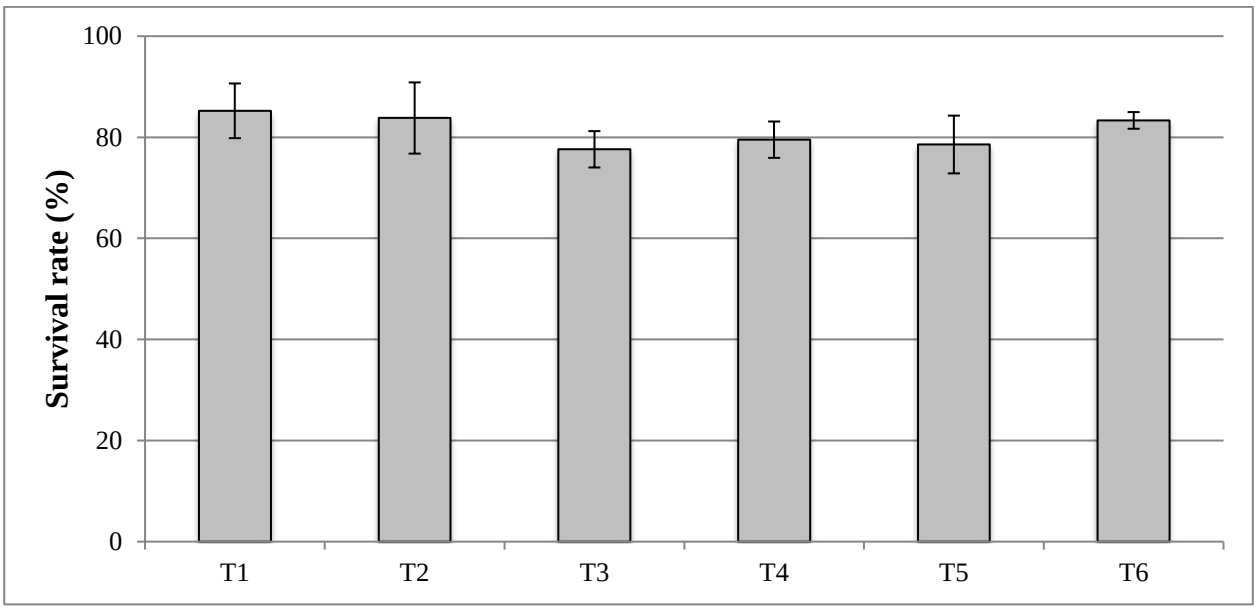


Figure 5.5 Percent survival rate of different treatments at the end of the trial (10 weeks) of experimental feedings. Presented values are means of triplicates ± standard error of mean.

5.4.2 The intestinal microbial count and probiotic monitoring in juvenile tilapia

At the beginning of the trial, sampled tilapia cultivable intestinal counts on EMA, BA and TSA were found to be log 1.1 to 3.9, 1.6 to 4.1 and 5.2 to 6.5 cfu.g^{-1}, respectively. The cultivable levels on the same media at the trial mid point and end point were not significantly different between the groups (Table 5.9).

At the mid-trial and the trial ending, both *Bacillus* and *P. acidilactici* probiotics were not detected by PCR using specific primers- in the GIT of all probiotic groups (T1, T2, T3, T4 and T5) and the control group (T6). However, *Enterobacter* probiotic was only detected in one sample in the T6 group (Figure 5.6).

Table 5.9 Log of cultivable microbial loads (log cfu.g^{-1}) in different media of the tilapia GI of each treatment fed supplemented probiotic. Presented values are means of duplicates ± standard error of mean.

Treatments	MRS-A		EMA		BA		TSA	
	Week 5	Week 10	Week 5	Week 10	Week 5	Week 10	Week 5	Week 10
T1	0.84±0.94	0.08±0.07	5.17±0.12	5.29±0.09	5.46±0.22	5.36±0.05	6.47±0.02	6.74±0.10
T2	0.91±0.44	0.72±0.48	5.50±0.15	5.27±0.08	5.55±0.15	5.35±0.18	6.58±0.29	6.59±0.17
T3	1.36±0.26	0.34±0.33	5.45±0.12	5.31±0.11	5.57±0.35	5.48±0.28	6.63±0.21	6.64±0.20
T4	0.60±0.73	1.19±0.46	5.35±0.08	5.21±0.04	5.44±0.04	5.33±0.15	7.05±0.23	6.62±0.25
T5	2.18±0.55	0.51±0.44	5.64±0.48	5.15±0.05	5.85±0.17	5.34±0.13	6.75±0.38	6.58±0.19
T6	0.87±0.64	0.61±0.84	5.77±0.39	5.37±0.26	5.61±0.12	5.24±0.06	6.89±0.37	6.59±0.18

5.4.3 Microscopic studies

The intestinal morphology of tilapia samples fed each of different diets was examined by light microscopy at the trial mid point and the trial ending (Figure 5.7 & 5.8). A simple columnar epithelium with mucosal folds were extended into the intestinal lumen was observed in samples. Each mucosal fold consisted of lamina propria, surrounded by a polarised layer of enterocytes interspersed by goblet cells and intraepithelial leucocytes. No significant differences of the abundance of goblet cells between treatments at the mid-trial and the end of the trial were observed ($P > 0.05$) (Figure 5.9). Goblet cells observed to be increasing following the time studies, which found to be 1941±692 (n=5), 2447±564 (n=18) and 2619±673 (n=18) cells.mm^{-2} at the initial trial, the mid-trial and the trial ending, respectively.

TEM micrographs were used to assess the morphology of the intestinal microvilli and microvilli parameters at the mid point (Figure 5.10) and the end point of the trial (Figure 5.11). Samples revealed well-formed, long, intact microvilli on the apical surfaces of enterocytes from all treatment groups. At the initial study, microvilli length was to be 0.588±0.049 μm, 0.055±0.009 μm of microvilli width, 10.919±2.194 of the proportion of microvilli length/ width and 0.106±0.021 μm^2 of microvilli areas. Significant differences ($P<0.05$) of microvilli length, width, the proportion of

length/width between treatments found at the mid-trial and microvilli width, the proportion of length/width and the proportion of microvilli length/ width between treatments found at the trial ending (Table 5.10). At the end of the trial, microvilli width was found the highest in T5, T3 and T4 and the lowest in T1 and length/width proportion was observed the highest in T1 and the lowest in T5. Furthermore, microvilli area was found the highest in T3, T4 and T1 and the lowest in T2. Microvilli properties seemed to be increased following the time up.

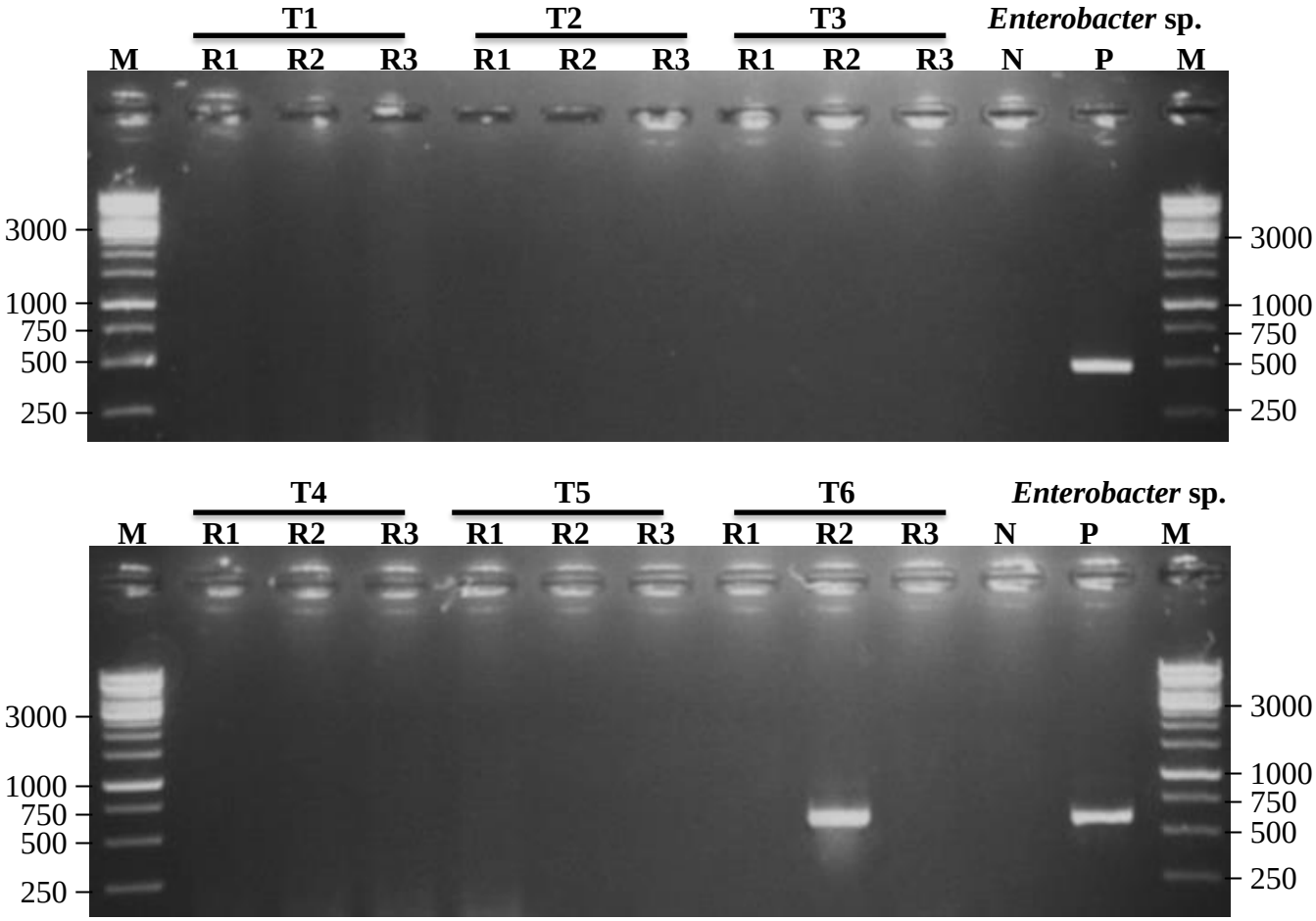


Figure 5.6 Probiotic monitoring using *Enterobacter* primer to detect probiotic colonization in the larval intestine at 10 weeks (M=100 bp plus DNA marker (Fermentas); N=Negative control (pure sterile water used as DNA template) and P=Positive control (Positive probiotics as used probiotic DNA templates); T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group).

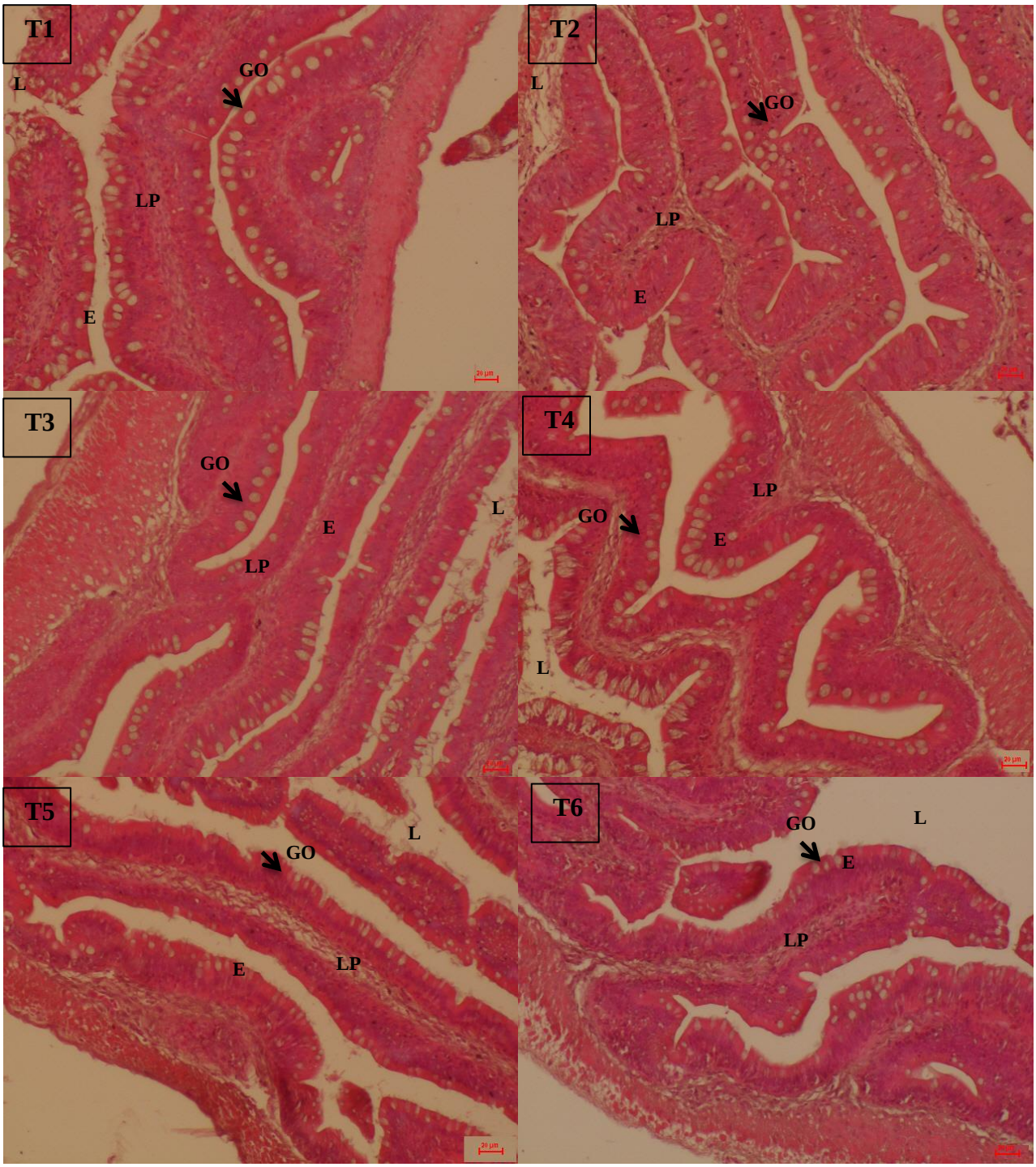


Figure 5.7 Light micrographs of the mid-intestine (H&E staining) of tilapia in different groups after feeding probiotic at 5 weeks (L=lumen, LP= lumina propria, E=epithelia, GO=goblet cells; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=20 µm.

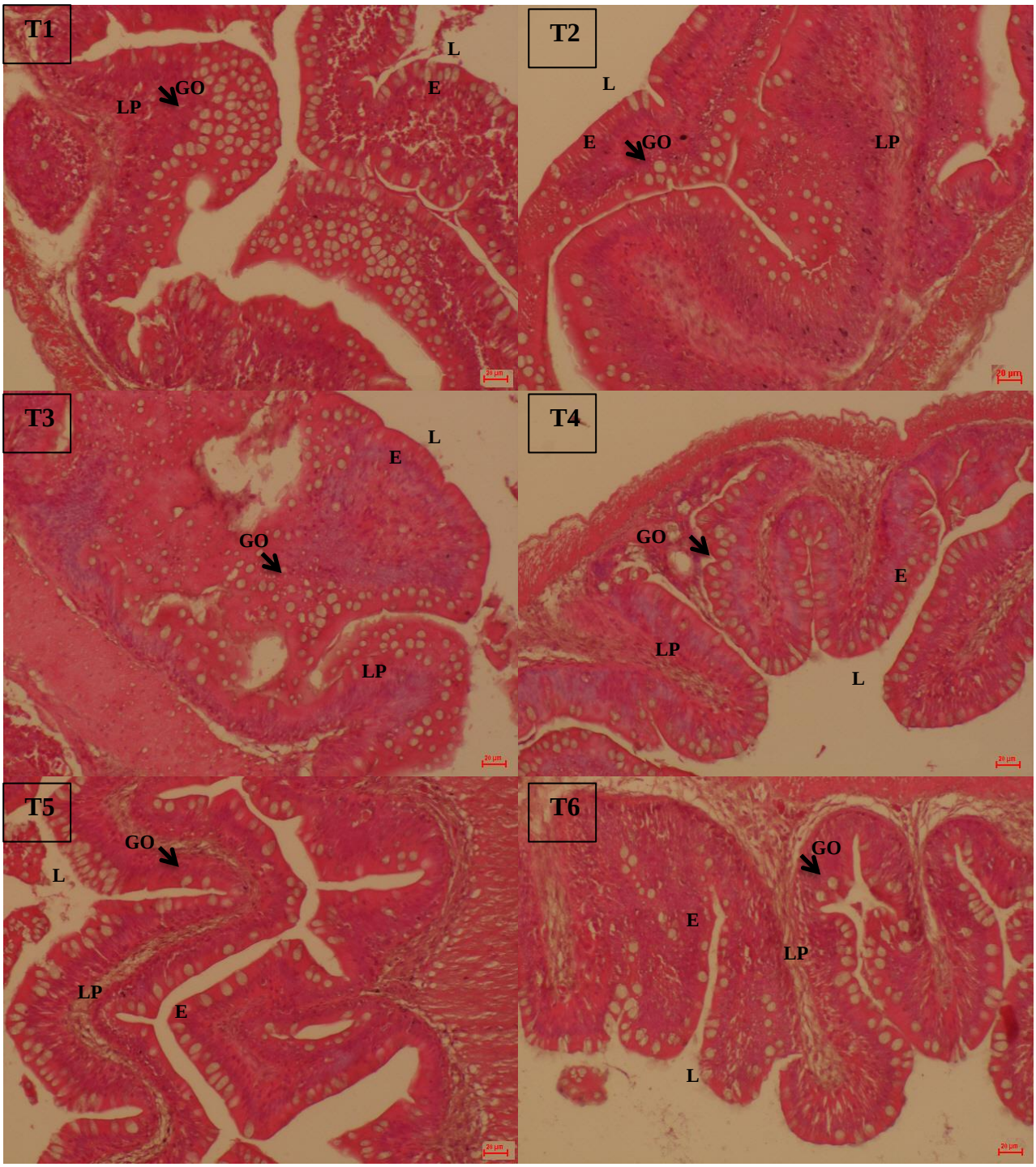


Figure 5.8 Light micrographs of the mid-intestine (H&E staining) of tilapia in different groups after feeding probiotic at 10 weeks (L=lumen, LP= lumina propria, E=epithelia, GO=goblet cells; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=20 μm.

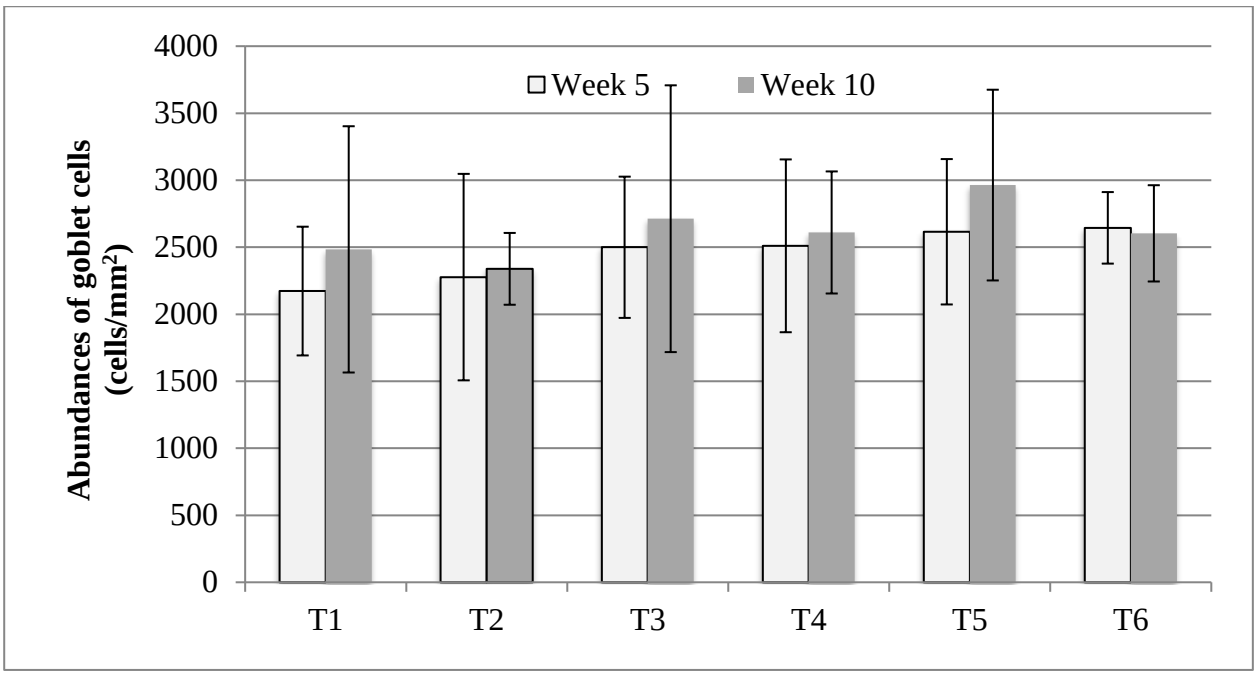


Figure 5.9 Abundances of goblet cells fed different treatments at the mid-trial (5 weeks) and the end of the trial (10 weeks). Presented values are means of triplicates ± standard error of mean.

The SEM micrographs at the trial mid point (Figures 5.12) and the end of the trial (Figures 5.13) clearly revealed complex mucosal folds and packed microvilli on the apical surfaces, with minor residues of mucus and digesta. Bacteria-like cells were also observed adhering to the mucosal epithelium, which were presumably autochthonous bacteria of the tilapia intestine. Several bacterial phenotypes (rod-shape and cocci-shape) were observed but no qualitative changes in abundance or colonization patterns were observed.

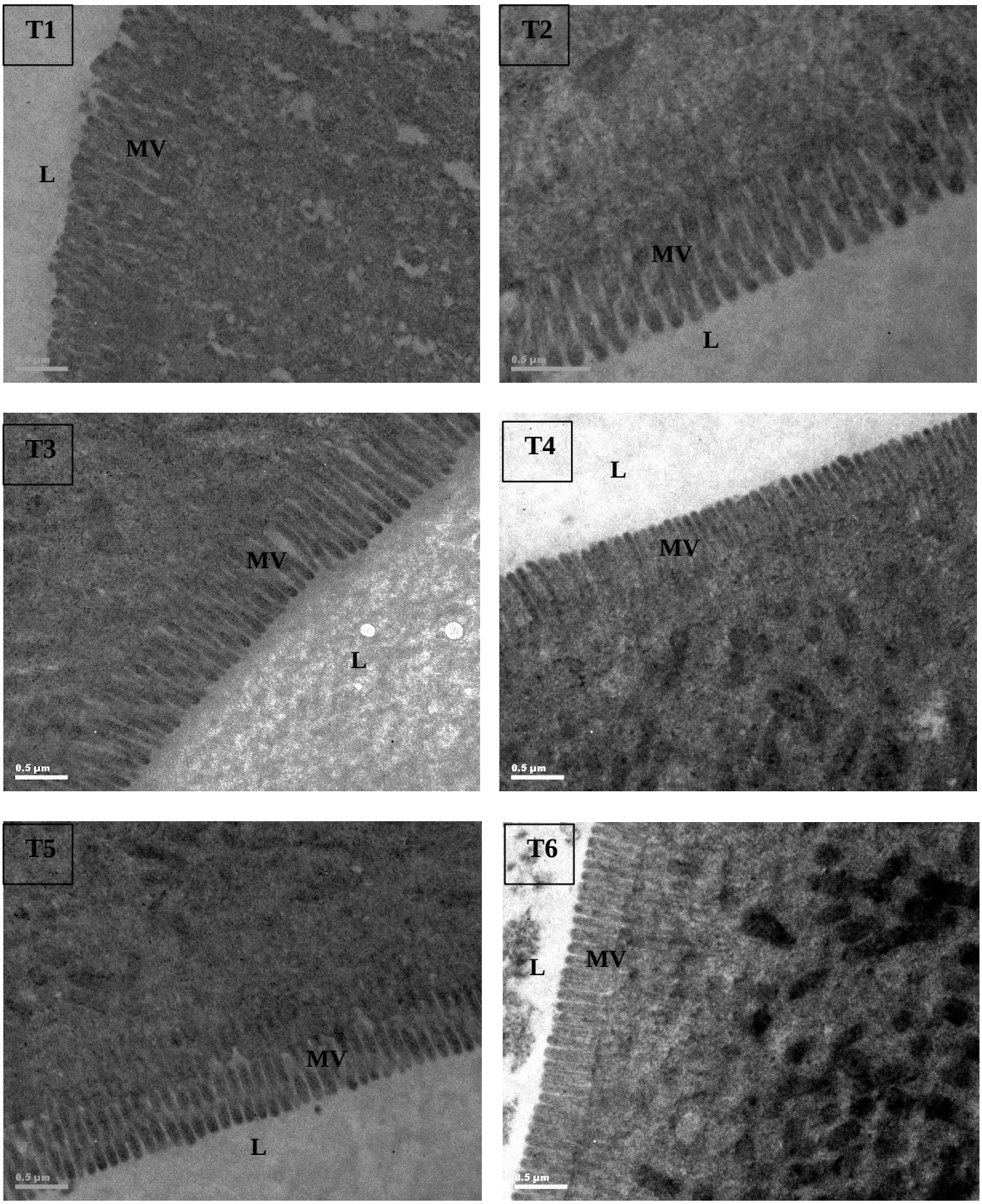


Figure 5.10 Transmission micrographs of microvilli of the mid-intestine of tilapia in different groups after feeding probiotic at 5 weeks (MV= microvilli; L= lumen; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=0.5 µm.

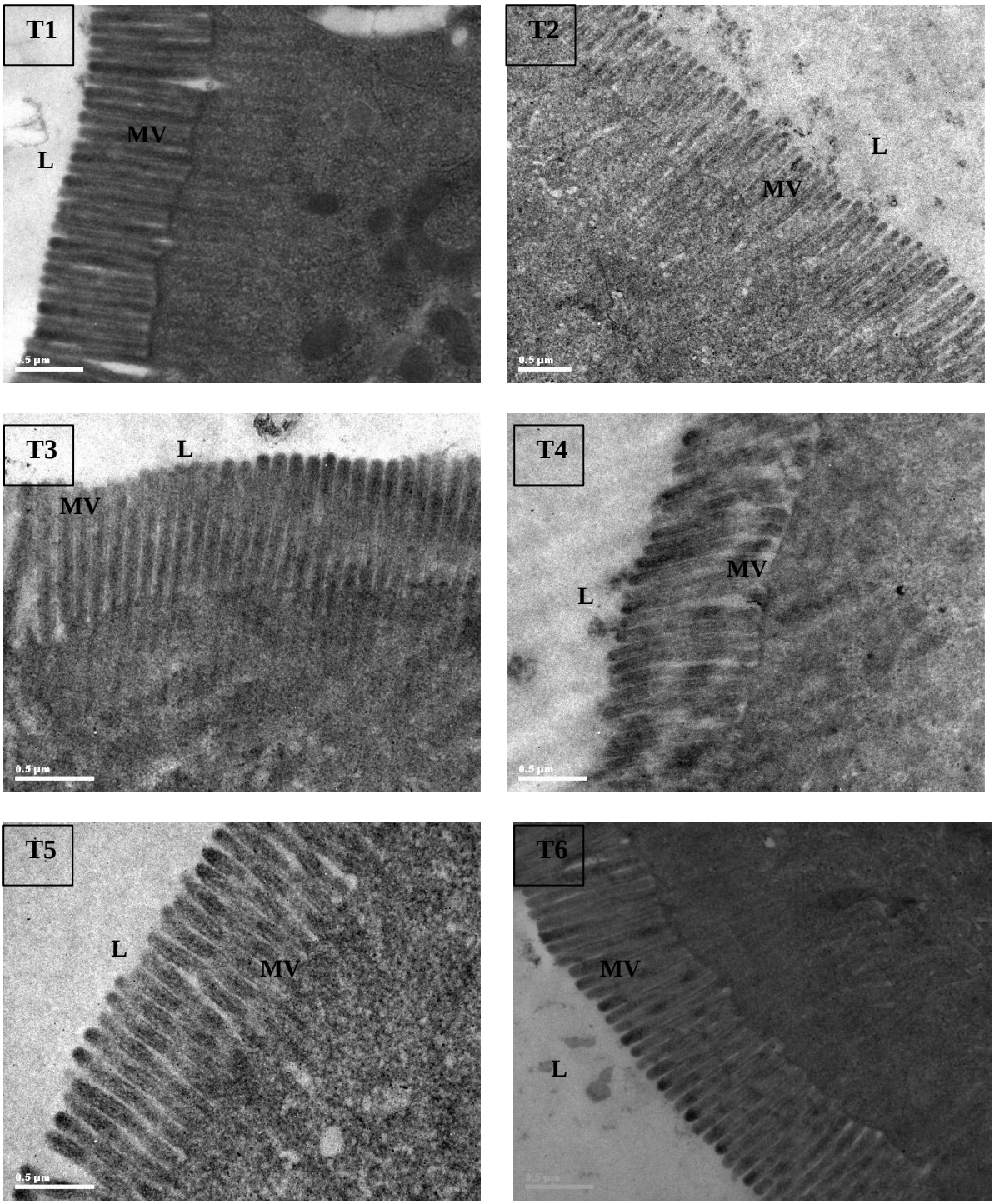


Figure 5.11 Transmission micrographs of microvilli of the mid-intestine of tilapia in different groups after feeding probiotic at 10 weeks (MV= microvilli; L= lumen; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group); scale bar=0.5 μm.

Table 5.10 Quantitative data of microvilli of the mid-intestine of tilapia samples of each treatment fed supplemented probiotic (mean ± standard error of mean).

Treatments	Lenght (µm)		Width (µm)		Length/Width		Area (µm^2)	
	Week 5	Week 10	Week 5	Week 10	Week 5	Week 10	Week 5*	Week 10
T1	0.875±0.117^{a}	0.933±0.038	0.071±0.009^{b}	0.079±0.010^{c}	12.480±1.962^{a}	12.182±1.554^{a}	0.200±0.042	0.236±0.032^{a}
T2	0.641±0.039^{a}	0.766±0.049	0.094±0.008^{a}	0.086±0.010^{ab}	6.878±0.714^{c}	9.176±1.301^{d}	0.197±0.023	0.213±0.029^{b}
T3	0.779±0.048^{a}	0.847±0.053	0.079±0.008^{b}	0.091±0.008^{a}	10.035±1.165^{b}	9.420±0.835^{c}	0.198±0.023	0.248±0.031^{a}
T4	0.621±0.040^{ab}	0.890±0.047	0.080±0.011^{b}	0.086±0.010^{ab}	8.029±1.161^{bc}	10.684±1.226^{bc}	0.161±0.023	0.243±0.034^{a}
T5	0.773±0.061^{a}	0.704±0.040	0.087±0.007^{a}	0.101±0.008^{a}	8.939±1.028^{b}	7.000±0.658^{e}	0.218±0.025	0.233±0.022^{ab}
T6	0.484±0.035^{ab}	0.852±0.054	0.091±0.010^{a}	0.082±0.011^{b}	5.393±0.791^{c}	11.389±2.071^{b}	0.151±0.019	0.216±0.035^{ab}

Significant differences (P<0.05) between treatments in each week are denoted by different superscripts in each column.

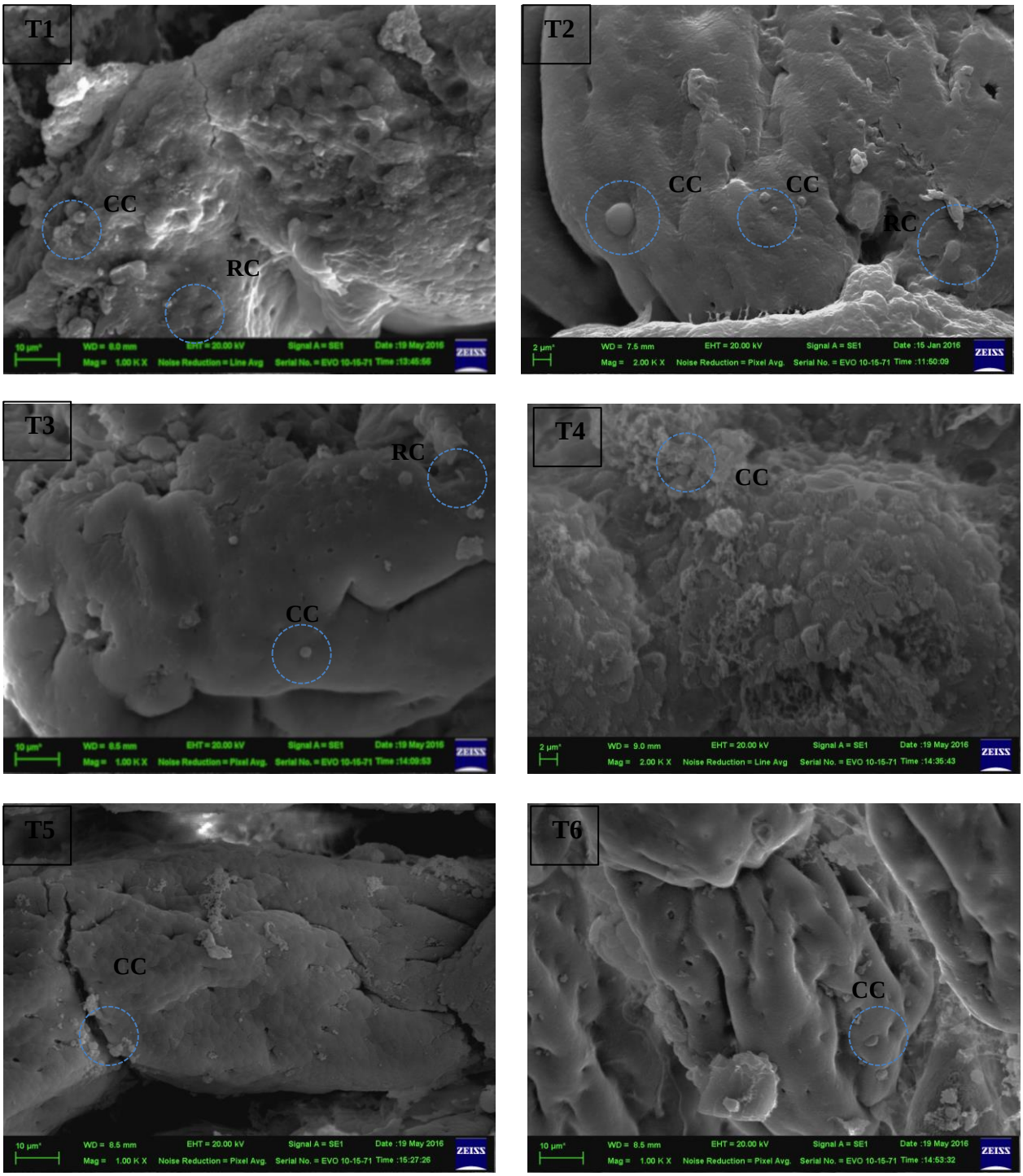


Figure 5.12 Scanning micrographs monitored bacterial colonization of the mid-intestine of tilapia in different groups after feeding probiotic at 5 weeks (CC=cocci-like-cell, RC=rod cell; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group; scale bar=10 µm (T1, T3, T5 & T6); scale bar=2 µm (T2 &T4).

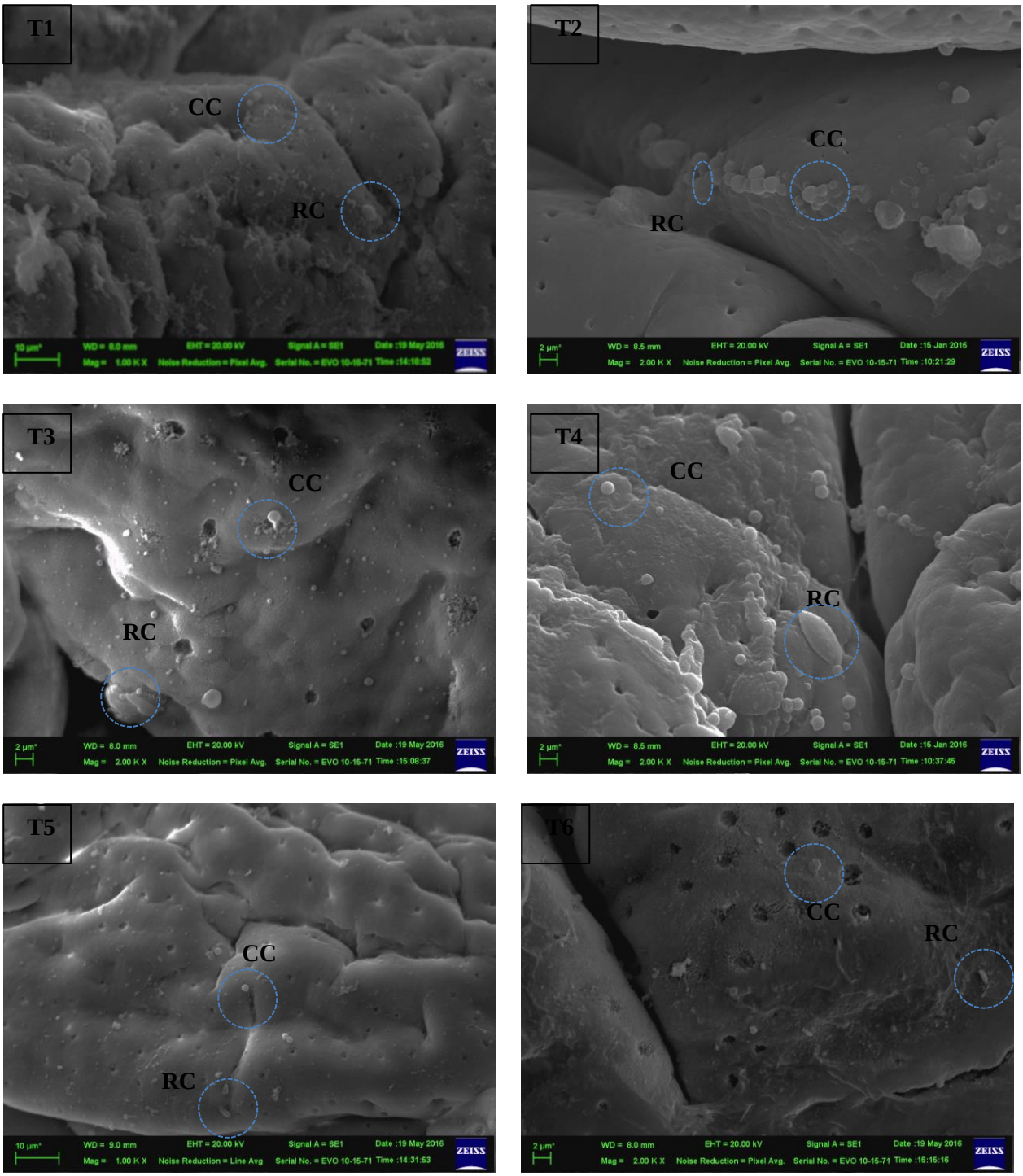


Figure 5.13 Scanning micrographs monitored bacterial colonization of the mid-intestine of tilapia in different groups after feeding probiotic at 10 weeks (CC=cocci-like-cell, RC=rod cell; T1= *Bacillus* sp. CHP02, T2=*Bacillus* sp. RP01, T3=*Bacillus* sp. RP00, T4=*Enterobacter* sp. NP02, T5=*P. acidilactici* and T6= the control group) scale bar=10 μm (T1 & T5); scale bar=2 μm (T2, T3, T4 & T6).

5.4.4 Stress inductions

5.4.4.1 Pathogenic induction

No significant differences between treatments of plasma cortisol levels was observed *A. hydrophila* challenged fish fed the different treatments 24 hrs after the IP challenge (Figure 5.14), whereas, significant differences of plasma glucose (Figure 5.15) and osmolality levels (Figure 5.16) were detected. A high level of plasma cortisol was observed in the T6 group, while a low level was observed in the T4 group. Stressed fish in T2 displayed a low level of plasma glucose, which was significantly lower than that of T3, T4 and T5 fish. In addition, a level of plasma glucose was also lowest in T2 fish and was significantly lower than T1 and T5 fish. The survival rates (Figure 5.17) after injecting pathogen into the IP cavity of fish fed each probiotic (T1, T2, T3, T4 and T5) and without probiotic (T6) was low after 7 days, ranging from 2 to 10% with no significant differences between the treatments (P>0.05). No mortalities occurred in the negative control groups (injected with sterile 0.85% NaCl)

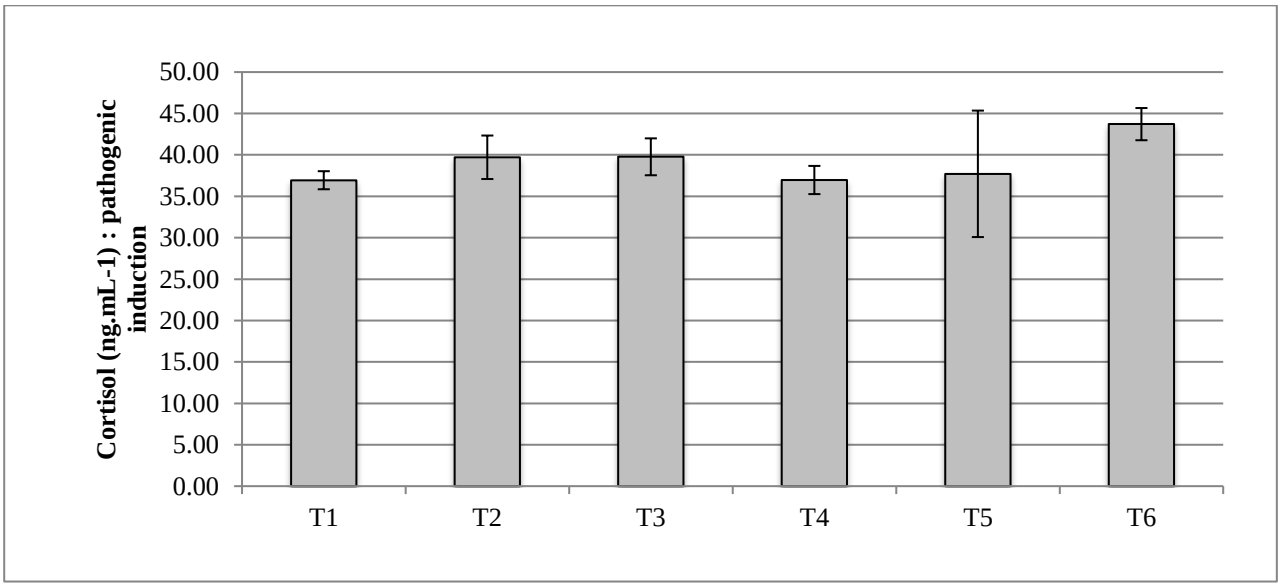


Figure 5.14 Plasma cortisol concentrations of fish fed different diets for 10 weeks and induced stress condition by using *A. hydrophila* injection. Presented values are means of triplicates ± standard error of mean.

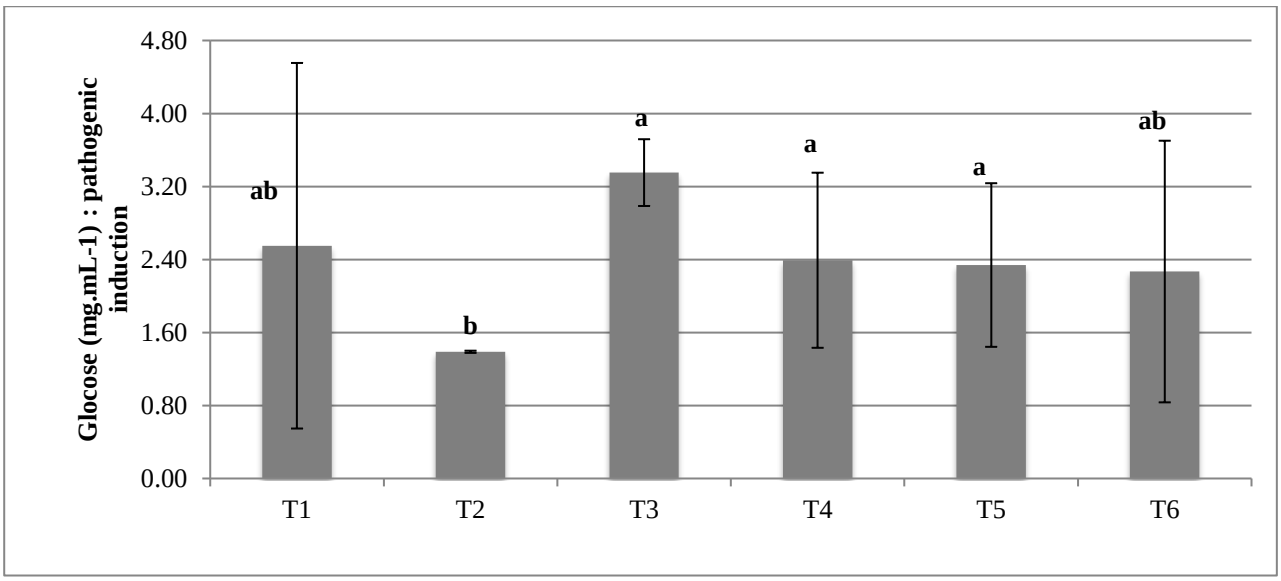


Figure 5.15 Plasma glucose concentrations of fish fed different diets for 10 weeks and induced stress condition by using *A. hydrophila* injection. Presented values are means of triplicates ± standard error of mean. Significant difference (P<0.05) between treatments denotes by different superscripts.

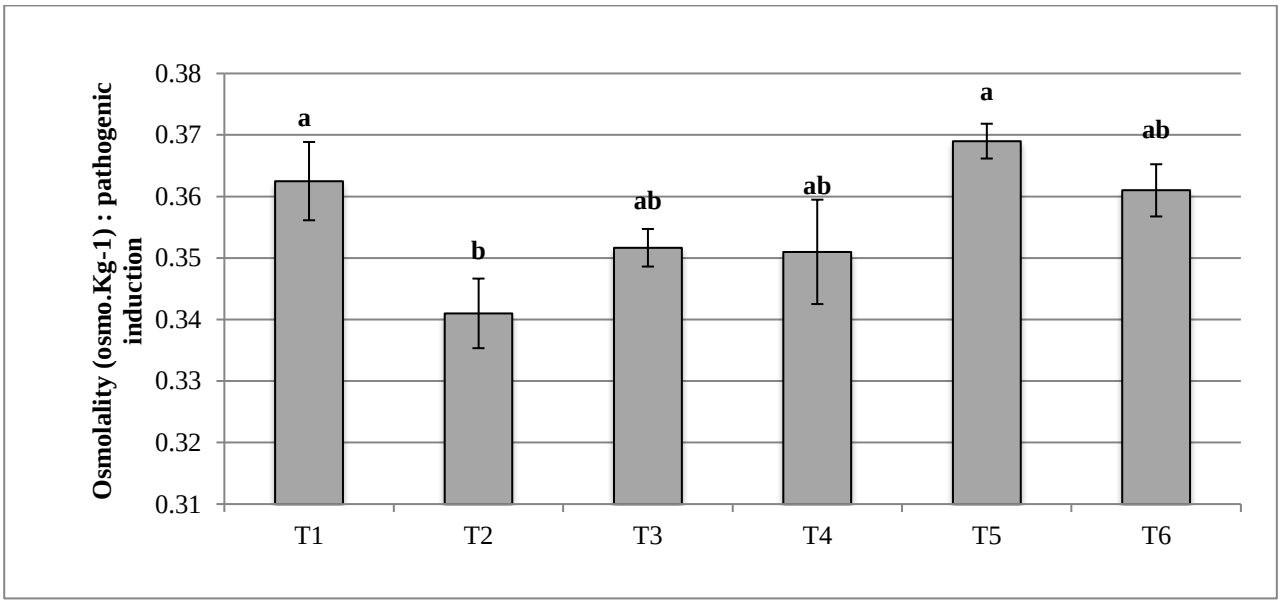


Figure 5.16 Plasma osmolality concentrations of fish fed different diets for 10 weeks and induced stress condition by using *A. hydrophila* injection. Presented values are means of triplicates ± standard error of mean. Significant difference (P<0.05) between treatments denotes by different superscripts.

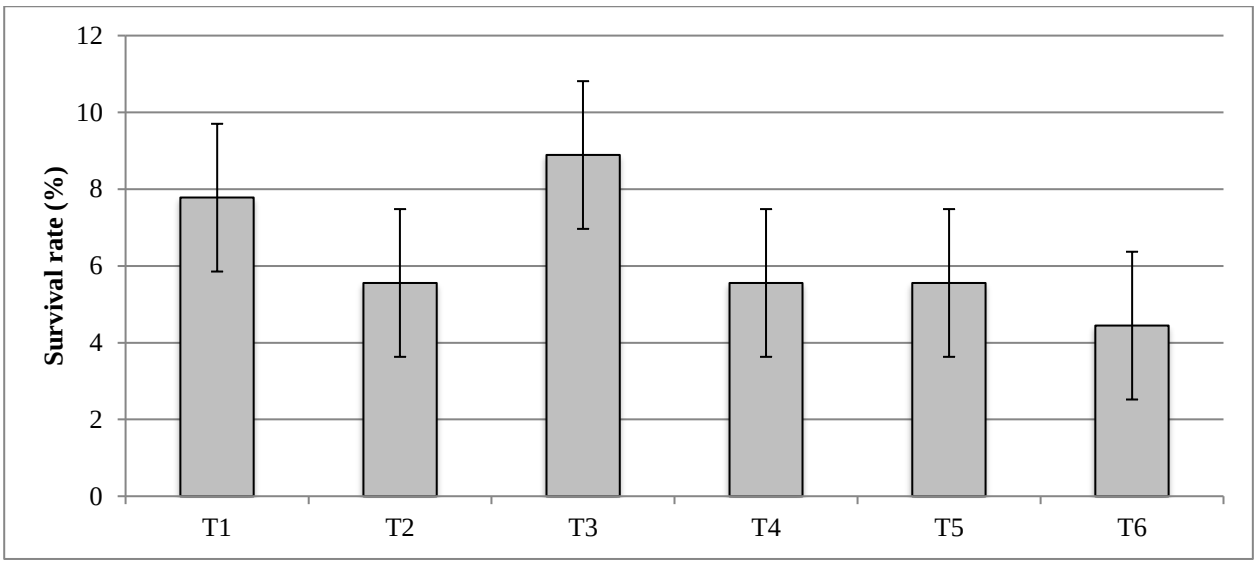


Figure 5.17 Survival rates of fish fed different diets for 10 weeks and induced stress condition by using *A. hydrophila* injection after monitoring for 7 days. Presented values are means of triplicates ± standard error of mean.

5.4.4.2 Thermal shock

The level of plasma cortisol in stressed fish displayed a significant difference ($P<0.05$) between fish fed different diets (Figure 5.18). The highest of cortisol level was detected in the T3 fed fish, which was significantly higher than T4 fed fish, which displayed the lowest cortisol levels and were significantly lower than that of T1 fed fish as well as T3fed fish. Plasma glucose in fish stressing of all treatments displayed no significant differences (Figure 7.19). Plasma osmolality in stressed samples (Figure 5.20) of treatments displayed significant differences with the levels of T4, T5 and T6 being significantly higher than T3, T2 and T1. The lowest osmolality was observed in T1, which was significantly different from all of the other groups. The thermal challenge did not cause any mortality in any of the treatment groups.

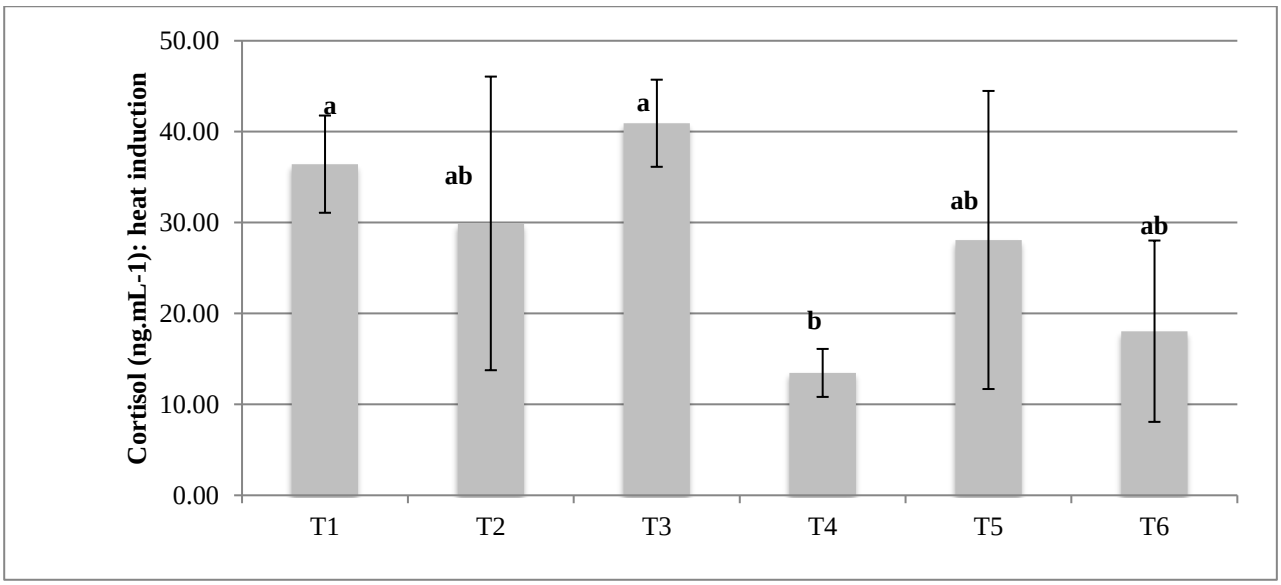


Figure 5.18 Plasma cortisol concentrations of fish fed different diets for 10 weeks and induced stress condition by thermal induction. Presented values are means of triplicates ± standard error of mean. Significant difference ($P<0.05$) between treatments denotes by different superscripts.

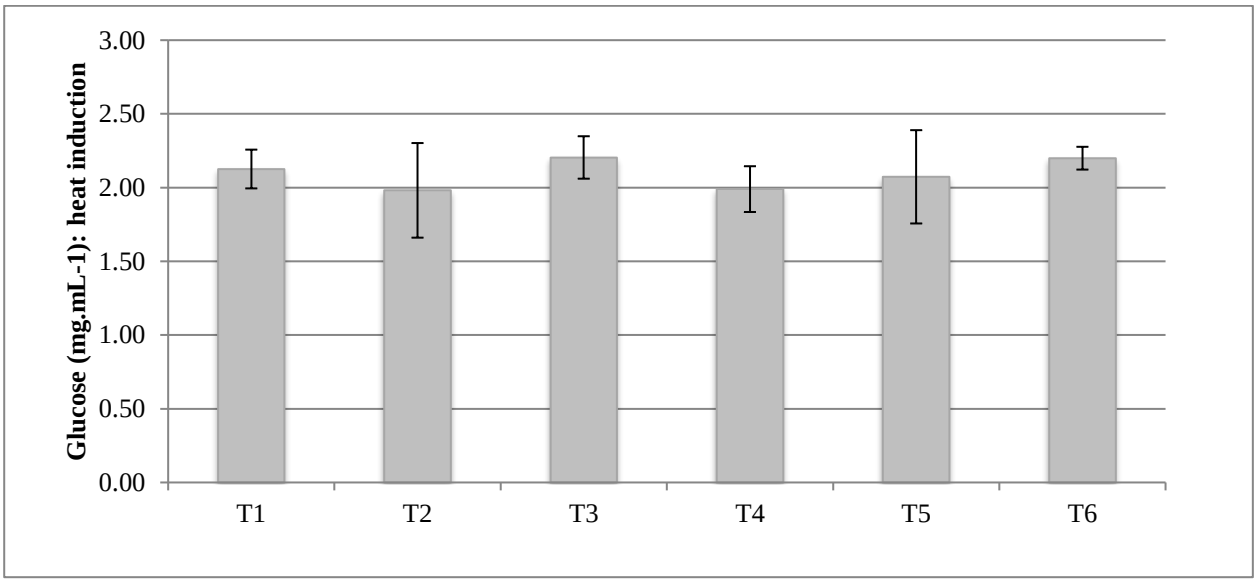


Figure 5.19 Plasma glucose concentrations of fish fed different diets for 10 weeks and induced stress condition by using thermal induction. Presented values are means of triplicates ± standard error of mean.

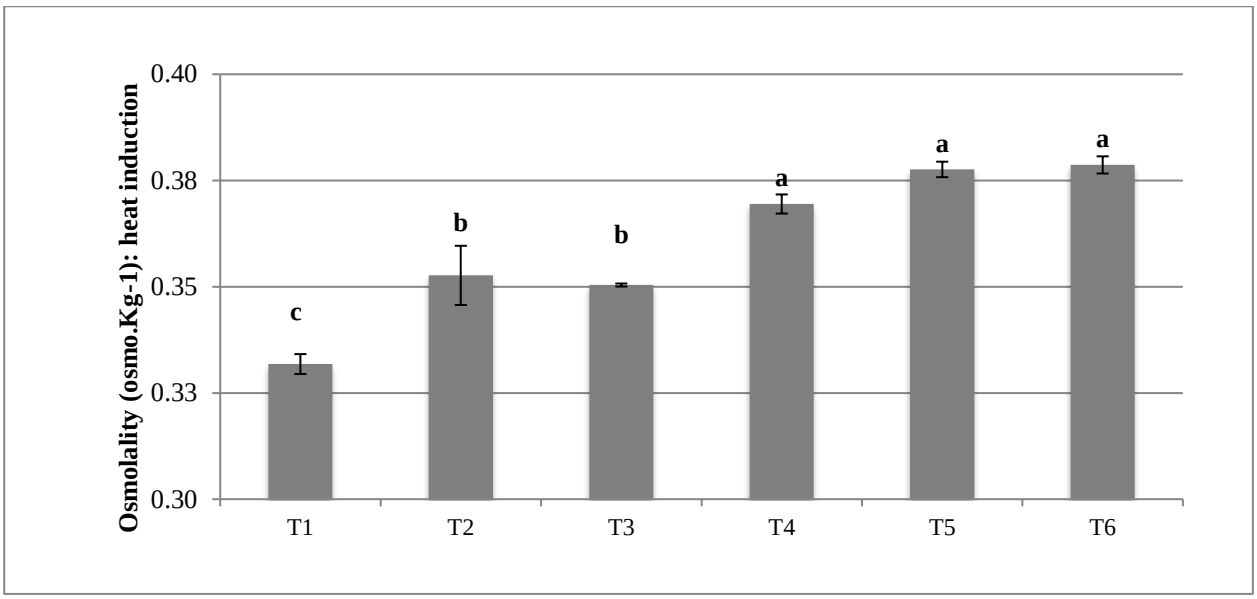


Figure 5.20 Plasma osmolality concentrations of fish fed different diets for 10 weeks and induced stress condition by using thermal induction. Presented values are means of triplicates ± standard error of mean. Significant difference ($P<0.05$) between treatments denotes by different superscripts.

5.5 Discussion

The ability of probiotic candidates, three strains of *Bacillus* spp. (CHP02, RP01 and RP00), one strain of *Enterobacter* NP03, was evaluated in comparison with a commercial probiotic (*P. acidilactici*) and a control group. In the present study, dietary probiotic concentrations of $10^{6\text{-}7}$ cfu.g^{-1} were fed to tilapia juveniles (6.9 to 7.1 g) for 10 weeks. Previous studies reported that a single dose of probiotic candidates as *B. amyloliquefaciens*, *B. firmus*, *B. pumilus*, *B. subtilis*, *Citro. freundii*, *L. acidophilus*, *Lactobacillus* sp. and *P. acidilactici* at concentrations of $10^{6 \text{ to } 12}$ cfu.g^{-1} diets have been supplemented in tilapia feed for evaluating tilapia having 5.2 to 9.1 g of mean weights and rearing for two to thirty-four weeks (Aly *et al.*, 2008a,b&c; Standen *et al.*, 2013).

In the present study, during the experiment, some parameters such as increasing weights, weight gains, increasing lengths, SGR, ADG and K factor displayed significant differences, however, at the

end of the trial no significant differences between in growth performance metrics were observed between the groups. The high ability of probiotics on growth performances may focus FCR parameter to reveal an amount of feed intake and a cost per yield. The *Bacillus* probiotic candidates in this study were not detected in the intestine 24hrs after cessation of feeding, and contrary to Chapter 4, did not affect FCR or other growth parameters. These finding are agreements that non-improvements on FCR of probiotics such as *P. acidilactici* (2.81×10^6 cfu. g^{-1} diet) fed tilapia for six weeks (Standen *et al*., 2013), *B. subtilis* (5×10^6 cfu. g^{-1} diet**)** fed tilapia for 12 weeks (Telli *et al*., 2014) and mixed probiotics (*B. subtilis*, *S. cerevisiae* and *A. oryzae*) fed tilapia for 4 weeks (Iwashita *et al*., 2015). The positive effect on FCR of probiotics such as a commercial probiotic (Biogens: *B. subtilis* Natto (not less than $6 \times 10^7.g^{-1}$) and the other components) fed tilapia for 17 weeks (EL-Haroun *et al*., 2006) and *B. amyloliquefaciens* (10^8 cfu. g^{-1} diet) fed tilapia 61 days (Ridha and Azad, 2012). It is evident from the results of the present study and those of Chapter 4 that the efficacy of probiotics can be dependent on life stage. This should be further explored in future studies, and probiotic concentrations of more than 10^{6-7} cfu.g^{-1} should be studied.

Several articles reported that tilapia feeding probiotics displayed 90% survival rate, which did not differ between probiotic groups and the control group (Standen *et al*., 2013; Telli *et al*., 2014; Hamdan *et al*., 2016). Similar result was observed in the present study, with survival rates of approximately 80%.

Microbial loads and probiotic identifications in the fish intestine are routinely studied to reveal the potential of probiotics. Microbial abundance and activities in the intestine of fish also relate to enzymatic activities and nutritional digestibility, which may lead to improved growth performances (Balcazar *et al*., 2006). In the present study no significant differences of cultivable microbial abundances on the different media were detected from tilapia intestinal samples from the different treatments either at the trial mid point or end point. Similar results reported by other researchers (Standen *et al*., 2013; Iwashita *et al*., 2015). High microbial loads from all experimental groups

were present on TSA. The numbers of cultivable intestinal bacteria on BA in all treatments were similar to those on EMA. Moreover, *Bacillus* strains are usually observed in the intestine of tilapia and freshwater ecosystem (Al-Harbi and Uddin, 2004; Chantharasophon *et al*., 2011; Mohanty *et al*., 2011; Del'Duca *et al*., 2013; He *et al*., 2013). However, three strains of *Bacillus* candidates were not detected in the intestine. *P. acidilactici* colonies were not detected from any of the samples. *Enterobacter* were only detected in some samples after 10 weeks. Moreover, the intestinal length of tilapia was found vary 23 to 31 cm in the initial study, 31 to 51 cm at five weeks and 62 to 80 cm at ten weeks. Then, microbial loads and probiotic monitoring may be randomized from a whole intestine. A tiny sample of the fish intestine may be affected the results.

Microscopic studies, including both LM and TEM were used to observe potential histological changes and SEM was used to observe bacterial colonization in the intestine of the host. Hamdan *et al*. (2016) reported that the positive effect of probiotics (*Lac. plantarum* AH 78) on microvilli length in tilapia juvenile (24.5 g). However, no significant differences of goblet cells and microvilli parameters such as length, width, length/width proportion and area between the groups at the mid or end points of the trial have been reported by Standen *et al*. (2015) and Adeoye *et al*. (2016). However, SEM micrographs from the present study revealed to varieties of bacteria-like cells of various morphologies that had colonized the tilapia intestine of probiotic groups and the control group. No discernable differences between abundances or colonization patterns were apparent.

The potential of probiotics to modulate immunological parameters enhancing the health status (Cerezuela *et al*., 2012) has been reported. Probiotic fed fish have been reported to display higher cortisol and glucose levels than the control group (Telli *et al*., 2014; Iwashita *et al*., 2015). However, no significant differences of plasma osmolality were observed in *Lac. rhamnosus* fed tilapia reared at low density (Gonçalves *et al*., 2011). In the present study, stress was induced by both pathogenic injection and thermal shock, after feeding probiotic diets, in order to evaluate fish physiological responses and the IR index. No significant differences of plasma cortisol levels were

observed 24 hrs after infection by *A. hydrophila*. The differences in the level of glucose and osmolality were observed differences between probiotic groups, but no different from the control group. Probiotic groups of T1, T3, T4 and T5 not different from the control group and these groups were differently found from the T2. It showed the lowest level of glucose. Parameters of cortisol and osmolality were found different in stressed fish, inducing by thermal condition. Only T4 group displayed a lower cortisol level than the other probiotic groups and the control group, while the T1 was observed lower osmolality than the other groups. These findings may reveal fish fed probiotics displaying different responses to the acute conditions, both pathogen and temperature change, whilst fish fed probiotic may suppress low levels of plasma parameters than the control group. The variation of plasma parameters in these samples may possible be related potential probiotics and sources of fish samples. These selected probiotics are used to evaluate in the present study as the wide types of potential probiotics without processing of probiotic selection *in vitro* conditions. After Thailand having flood crisis in 2011, many farms were lost tilapia bloodstock and new brood stocks have been transferred from some public and private areas, which might affect high genetic variability.

Fish fed probiotics displayed enhanced resistance against pathogenic diseases due to modulations of non-specific immune responses (Hamdam *et al*., 2016). Pirarat *et al*., (2006) reported that fish fed probiotic *Lac. rhamosus* for two weeks displayed high survival rate for protecting fish from *E. tarda* pathogen. Aly *et al*., (2008a) used *B. pumilus* fed tilapia for 2 weeks and these fish displayed high survival rate resisting *A. hydrophila*. Iwashita *et al*., (2015) reported that mixed probiotics fed tilapia for five weeks provided to against *A. hydrophila* and *S. iniae*. Furthermore, fish fed probiotics (*B. pumilus* or commercial probiotic) for long-term period (8 months) displaying high resistance to pathogenic infection more than a short-term period of feeding (Aly *et al*., 2008c). In the present study, fish of all experimental groups displayed survival rates less than 10% after injecting a pathogen. It is clear in this scenario the mortality levels were too high to allow for a comparison of probiotic efficacy, which was what planned according to doses evaluated in

preliminary studies. The reason for the difference between the baseline mortality level and the preliminary experiment is not clear, though the results can be observed in all experiment groups (Figure 5.17) and thus, can still be credible.

In conclusion, the benefits observed with the autochthonous probiotics in Chapter 4 were not fully replicated in the current chapter. Probiotic groups were not the effect on growth performances, homeostatic states in the extreme conditions and survival rate in juvenile tilapia.

Chapter 6

General discussion and conclusions

The objectives of this thesis were to identify, evaluate and determine probiotic properties (multi-parameter) such as adherence with the intestinal epithelial cells of tilapia, adhesion to hydrocarbons, auto-aggregation, antibiotic resistance, blood hemolysis, bile salt and acid tolerances, and temperature exposures of the autochthonous bacteria originated from the intestinal of tilapia in *in vitro* trials (Chapter 3). Accordingly, bacteria were selected as high potential probiotic candidates by using the Z−scores. The potential of probiotic candidates were evaluated both tilapia fry (Chapter 4) and on-growing stage (Chapter 5). These objectives are represented in the overall protocols in Figure 1.7 (Chapter 1).

The results will be discussed in all experimental chapters, which begins with Chapter 3 about the screening and selection the potential probiotics *in vitro* assays. Probiotic properties of bacterial isolates were used to select a high potential of probiotic candidates by using a classicla methods as the Z-scores. This technique is combined with multi-parameter properties together with expecting the positive results in *in vivo* trials both in larval and juviniel stgaes of tilapia experiments, moreover, it will propose how to select probiotics for tilapia cultures.

Chapter 3, thirty-four bacterial colonies isolated from the intestine of tilapia; fifteen of these isolates antagonised the tilapia bacterial pathoginics *A. hydrophila* or/and *S. iniae*. The genomic identification of these isolates were displayed as seven strains of *Bacillus* spp. **(**RP00, RP01, CHP01, CHP02, RC00, RC01 and RC02), three strains of *B. cereus* **(**CHP00, NP01 and RP00), two

strains of *Enterobacter* spp. (NP02 and NP03), *Mac. caseolyticus* (CHP03), *Stap. arlettae* (CHP04) and *Stap. sciuri* (NP04)**.** The dominant cultivable bacteria of the tilapia intestine in this study was Bacillus species, which have phenotypes of rod-shape, spore forming, with granule in cell, and facultative anaerobes. Several *Bacillus* spp. strains such as *B. subtilis*, *B. pumilus* and *B. cereus* are often distributed in freshwater ecosystem (Mohanty, *et al*., 2011) and they were observed in the intestine of tilapia on several occasions (Al-Harbi and Uddin, 2004; Chantharasophon *et al*., 2011; He *et al*., 2013; Del'Duca *et al*., 2013).

In the present study, multiple parameters: antagonistic activity, cell-adhesive potentials, hemolytic activities, antibiotic resistance, pH and bile salt tolerances and specific growth rates were used for evaluating potential probiotics. These parameters are based on the review of literature in Table 1.2 (Chapter 1) as classical model of probiotic selection (Figure 6.1). At the same of this study, nine properties of potential probiotics were set to evaluate bacteria isolates having the purified stocks. The difference of probiotic selection was differed from several articles. Because of the condition of probiotic selection based on three groups of probiotic properties consisting of general parameters, safety parameters and survival parameters. General parameters included pathogenic antagonism and adhesion assays. Three parameters were divided into sub-parameters, which have different scores. Accordingly, the coefficient index was then calculated by using these scores, which had assumptions proposing in 3.3.5.9 the protocol to select probiotic candidates (Chapter 3), which yielded interesting results (Table 3.9 in Chapter 3).

Based on fifteen isolates, if probiotic selection based on antagonistic activities alone, ten isolates would have been classified as showing promise. If probiotic potential was based on antibiotic resistance, six isolates might be selected for further study. If the parameters for probiotic selection were based on antagonistic activities and hemolytic assays, then eleven isolates were selected for further study. If the parameter based on antagonistic activities, hemolytic assays and antibiotic resistance, only four isolates were selected for further studies. Such a restrictive approach would

have meant that some isolates having good potentials of many parameters could be, perhaps erroneously or unwisely, eliminated. Therefore, the approach used in the present study included a combined multi-parameter approach, using average mean of each parameter and the standard score (Z−score) was used to analyze these data for selecting potential probiotics (Table 3.8). Similarly, Vine *et al*., (2004) suggested selecting the potential of probiotics by using the ranking index (RI) by using able growth characteristics (lag-period and doubling-time) of isolates as an individual selection, while the standard score in this study is combined all parameters and isolates for calculation.

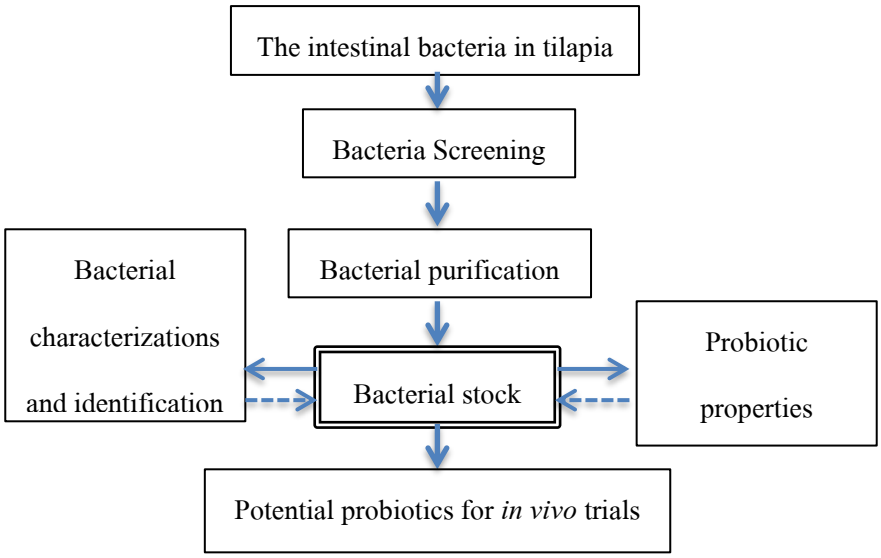


Figure 6.1 The classical model of probiotic selection.

According to the results from Chapter 3 screening of potential probiotics by using combined selection led to the identification of 4 isolates with positive Z scores that could be further studied *in vivo*. The benefit of such *in vitro* studies as a preliminary tool prior to *in vivo* studies reduces the number of fish used in research studies by refining the number of viable isolates worthy of testing *in vivo*, which in turn reduces costs.

The autochthonous probiotic candidates, consisting of three strains (CHP02, RP01 and RP00) of *Bacillus* spp. and *Enterobacter* sp. NP02 were evaluated in tilapia fry (Chapter 4) and on-growing stage (Chapter 5). In addition, the other groups were a commercial probiotic (*P. acidilactici*) as the positive control and a control group (without probiotic-feeding) as the negative control were used to compare the efficacy of the autochthonous probiotic candidates. The selected isolated have vary sources, which found *Bacillus* CHP02 originating from Chitralada strain in the closed system at KMITL, *Bacillus* RP00 and RP01 from red tilapia cultured in a pond and *Enterobacter* NP02 from tilapia reared in a pond.

Chapter 4 and 5 will be discussed together, these fish having different ages and sizing were both transport from AIT. In Chapter 4, tilapia fry without sex-reversal approximately having 81 mg of total weigh were used to evaluate the potential of probiotic selection, while tilapia weighted 7 g were used in Chapter 5. In fry stage, fish were fed six days a week to apparent satiation every 2 hours from 9.00 am to 5.00 pm and juvenile stage were fed three times per day at the rate of 10% biomass in the first week, 6% biomass in the second to the third weeks and then 4% biomass were used to feed fish until the end of the trial. Based on each rearing tanks were used to each probiotic for protecting the contamination in both trials. In this programme of research, significant improvements of growth performances were achieved with autochthonous probiotic feeding in fry (Chapter 4), while these benefits were not replicated in the on-growing trial (Chapter 5).

The effect of probiotic on 2 to 6 g tilapia were reported different findings. Nouh *et al*., (2009) used the mixed commercial probiotics (*B. subtilis* and *L. acidophilus*) and reported that probiotic feeding tilapia for one month could promote disease resistance and healthy fish. *Lac. acidophilus* supplemented feeding for 15 days have also been reported to improve survival rates during a pathogenic challenge (Villamil *et al*., 2014). A commercial *B. subtilis* probiotic and autochthonous probiotic (*Micro. luteus*) could provide growth performances improvements when fed to tilapia for 3 months (Soltan and El-Laithy, 2008; El-Rhman *et al*., 2009). Autochthonous LAB mixes in fish

feed and *Bacillus* spp. (originated in tilapia pond) adding in the rearing system, these can promote growth and high survival rate (Apún-Molina *et al*., 2009). A commercial probiotic (containing *Strep. faecium* and *Lac. Acidophilus*) has also displayed positive effects on growth performances in tilapia larvae (Lara-Flores *et al*., 2003). On the contrary, Shelby *et al*., (2006) reported that several commercial probiotics could not caused to support growth performances in tilapia larvae.

These are therefore not clear if the reduced probiotic efficacies are direct results relating to different life stages, or if they are caused indirectly, by the different rearing protocols necessitated for culturing different life stages. For example, probiotic candidates may have more easily and abundantly populated the rearing water and rearing environment the larvae given the more frequent feeding frequency, and the higher feed residence time in the water in Chapter 4 in comparison with the quick feeding fry exposed to fewer feeding periods in Chapter 5. Indeed, in the present studies, contradictory results were observed for probiotic recovery in the GIT between Chapters 4 and 5, which may support this speculative theory. The possible to evaluate the potential probiotics should be continuously mixed in rearing system for testing in growing stage.

The cultivated microbial loads in the intestine of tilapia fed different diets seemed to be similarly in both tilapia larvae (Chapter 4) and the growing stage (Chapter 5). A good recovery of probiotics in the GIT of probiotic feeding tilapia has reported by Bucio Galindo *et al*. (2009), Standen *et al*., (2013) and Iwashita *et al*., (2015). The discrepancy between these studies and that of the current study, particularly in regards to chapter 5, may be due to the dosage administered to the probiotic strain used. However, the fact that the tilapia in the present study were deprived of feed (and thus probiotic provision) for 24 hours prior to sampling is likely to be a key factor for the infrequent recovery of the probiotics. Moreover, Galindo *et al*., (2009) that probiotic persistence and recovery levels in the tilapia GIT are highest within 24 hours of feeding, with levels decreasing rapidly thereafter.

Stress inductions were used in this study by exposing pathogenic and thermal stressors after the post probiotic-feeding. Stressed fry (Chapter 4) were too small (3.95±0.356 g) to take a blood samples to monitor stress biomarkers, however, and blood samples were taken from juveniles (Chapter 5) to determine physiological stress responses (plasma cortisol, glucose and osmolality). The fish stress response has function stress hormones in progress to blood circulation, which raise cortisol and glucose levels (Reid *et al*. 1998) as react to the homeostatic situation (Iwama *et al*. 1999). Fish reared in stressful conditions may generally respond increasing gill permeability for exchanging ions and caused by plasma ionic losing (Cataldi *et al*., 2005). The differences of plasma cortisol and glucose in tilapia feeding probiotics displayed varying in each week (Iwashita *et al*., 2015). In the present study (Chapter 5), fish fed *Enterobacter* ENP02 displayed low cortisol after both pathogenic and thermal shock challenges. Fish fed *Bacillus* BRP02 displayed low glucose both pathogenic and thermal inductions, while plasma osmolality was differently occurrences both pathogenic injection and thermal shock. Results can suggest potential probiotics display no patterns both increased and decrease releasing of plasma parameters. It might be associated with probiotic strains and individual tilapia.

As Gonçalves *et al*. (2011) reported modulation of physiological stress responses that fish fed probiotics both under optimal and stress conditions, with decreased plasma cortisol levels in tilapia fed probiotics, while Telli *et al*. (2014) reported plasma cortisol and glucose levels of fish reared at different densities were not modulated by probiotic feeding. Conversely, El-Rhman *et al*. (2009) reported that glucose levels in probiotic groups lower than non-probiotic groups.

In conclusion, fifteen isolates from the intestinal of tilapia displayed to inhibit pathogens (*A. hydrophila* or/and *S. iniae*). The putative isolate was found in ten *Bacillus* spp. of fifteen bacteria. The combined multi-parameter approach and inclusion of ranking by Z-scoring was used to select high potentials of probiotic candidates, which found top three ranking autochthonous probiotic candidates as *Bacillus* sp. CHP02, RP01 and RP00. These strain contained good qualities and

favourable properties: (i) inhibition to pathogens, (ii) high adhesive potential to the tilapia epithelial cells, (iii) adhesive potential to hydrocarbons, (iv) auto-aggregations, (v) an antibiotic susceptibility, (vi) non-hemolytic activity, (vii) tolerance to 6% bile salts, (viii) resistance to pH 2, and (ix) acceptable growth at temperatures approve to tilapia farming.

These probiotic candidates (*Bacillus* sp. CHP02, RP01 and RP00), the fifth ranking scores as *Enterobacter* sp. NP02, a commercial probiotic (*P. acidilactici*) and the control group were evaluated in tilapia larvae. It appears that successful outcomes in fry tilapia depended on high volume of Z-scores. The most effective probiotic candidate was *Bacillus* sp. RP01, which improved average body weight, total weight gain, average daily growth, and specific growth rate in tilapia larvae. *Bacillus* sp. can colonise in the intestine of tilapia larvae after feeding for three weeks. The effective on growth performances of autochthonous probiotic than allochthonous probiotic is clear in fry stage. *In vivo* juvenile trial, the potential of autochthonous probiotics were not differed from the allochthonous probiotic and the control group. This study has shown the effective of the protocol to select probiotic as multi-parameter *in vitro* assays. High effectiveness of probiotic on tilapia culture may begin at the larval than growing stage.

Future studies should assess the followings: selective probiotic as the same of aquatic animals and then prove *in vivo* trials, which follow a range of probiotic dietary inclusion levels, supply via the rearing water, long term feeding trials with fish reaching market size, and assessment of immunological parameters. Moreover, high throughput sequencing to generate libraries or metagenomics to elucidate possible effects of probiotic feeding on the total microbial community (cultivable and non-cultivable) in the intestine are required study on the future.

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Appendix

Appendix 1: Morphological studies of bacterial selection

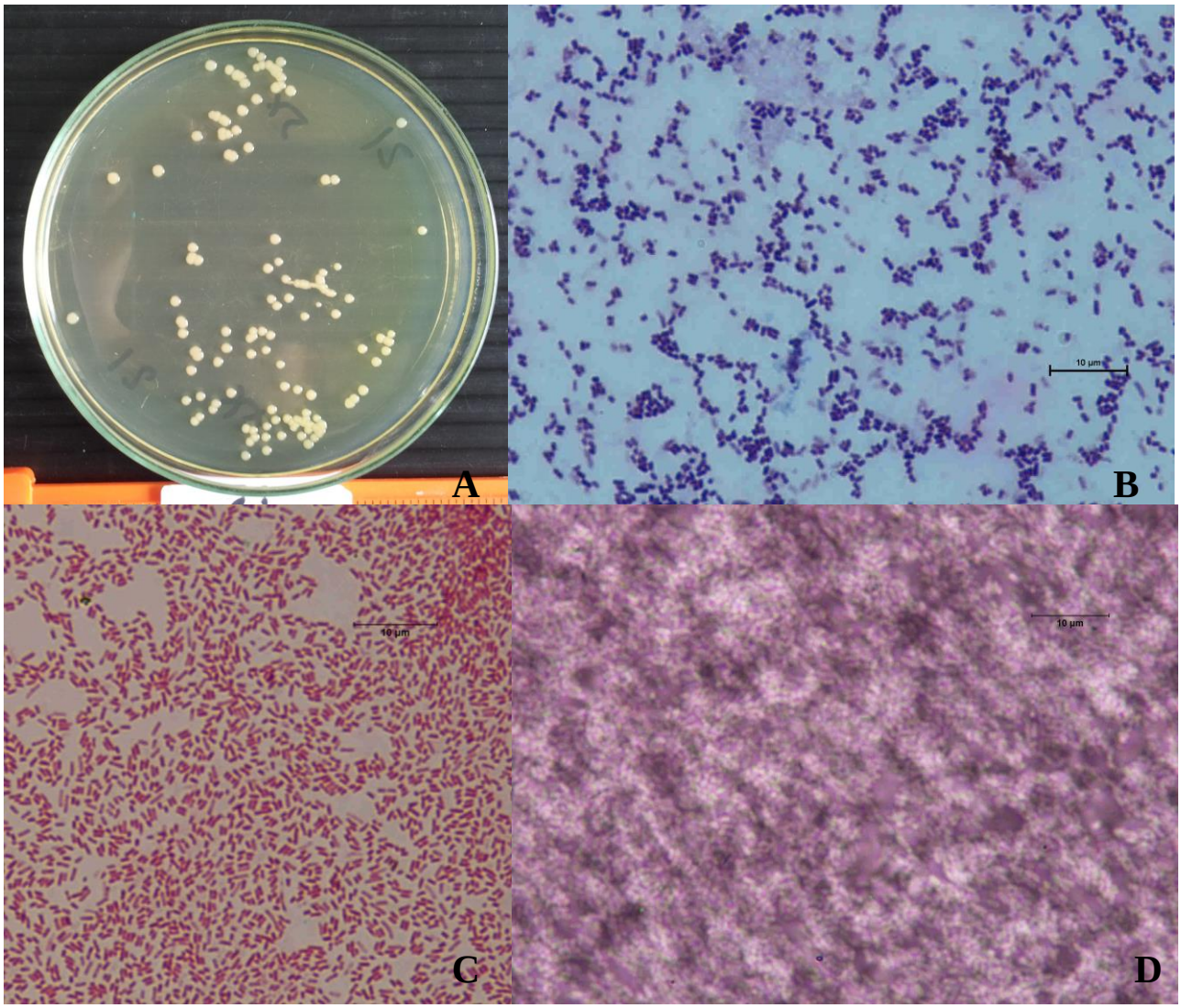

Figure A.1 *Bacillus* sp. CHP02; A: Morphology, B: Gram stain, C: Spore shape and D: Capsule.

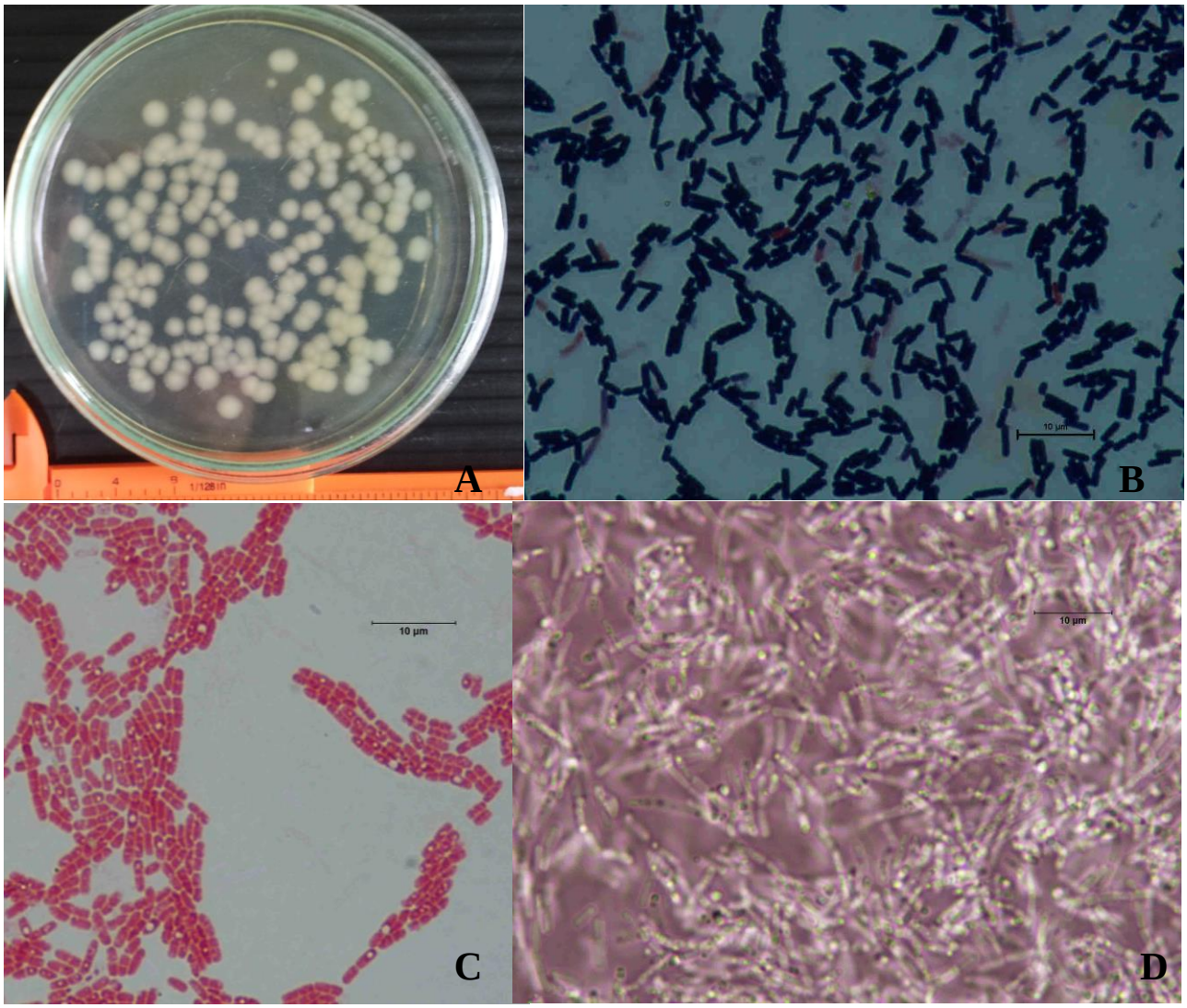

Figure A.2 *Bacillus* sp. RP01; A: Morphology, B: Gram stain, C: Spore shape and D: Capsule.

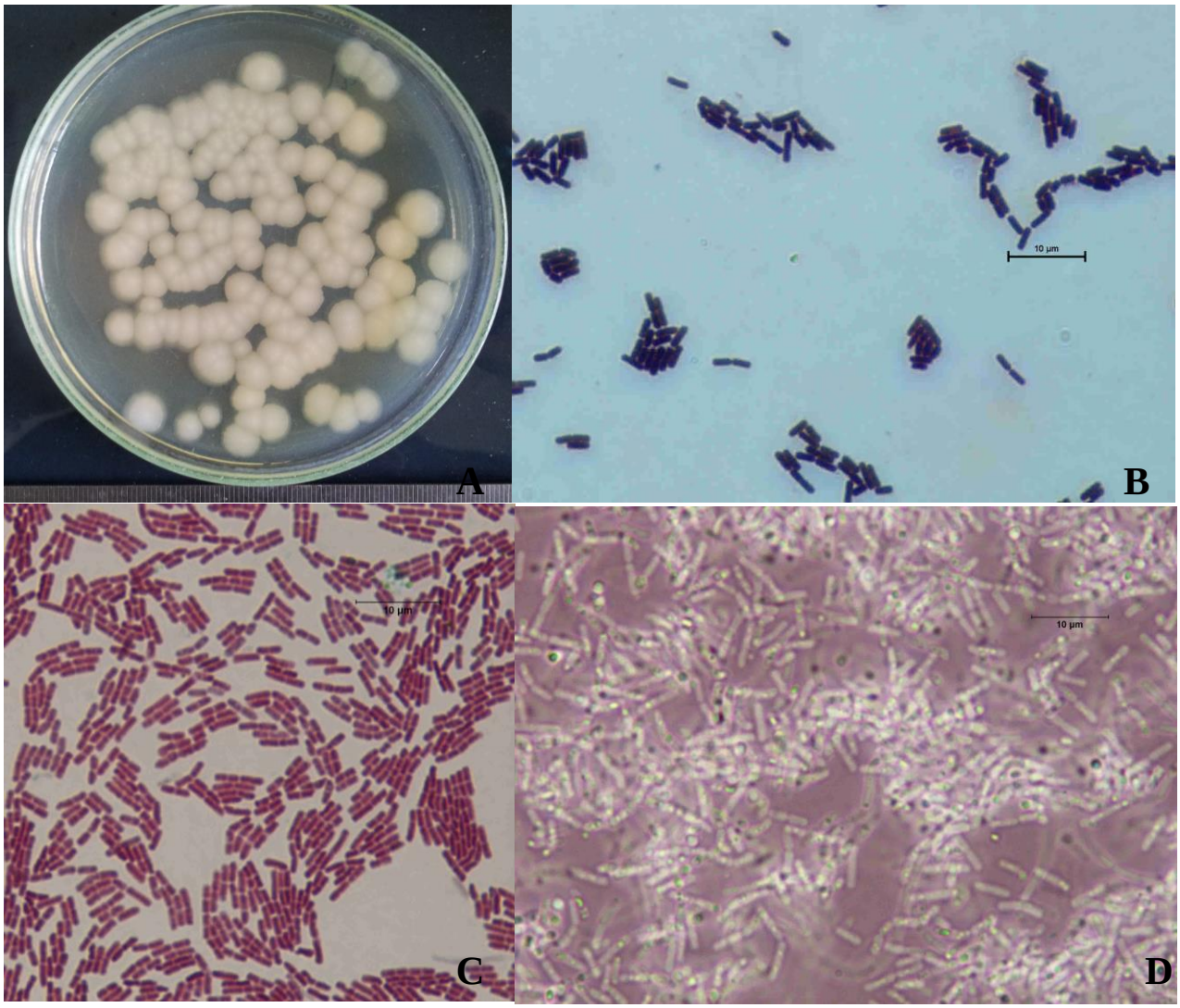

Figure A.3 *Bacillus* sp. RP00; A: Morphology, B: Gram stain, C: Spore shape and D: Capsule.

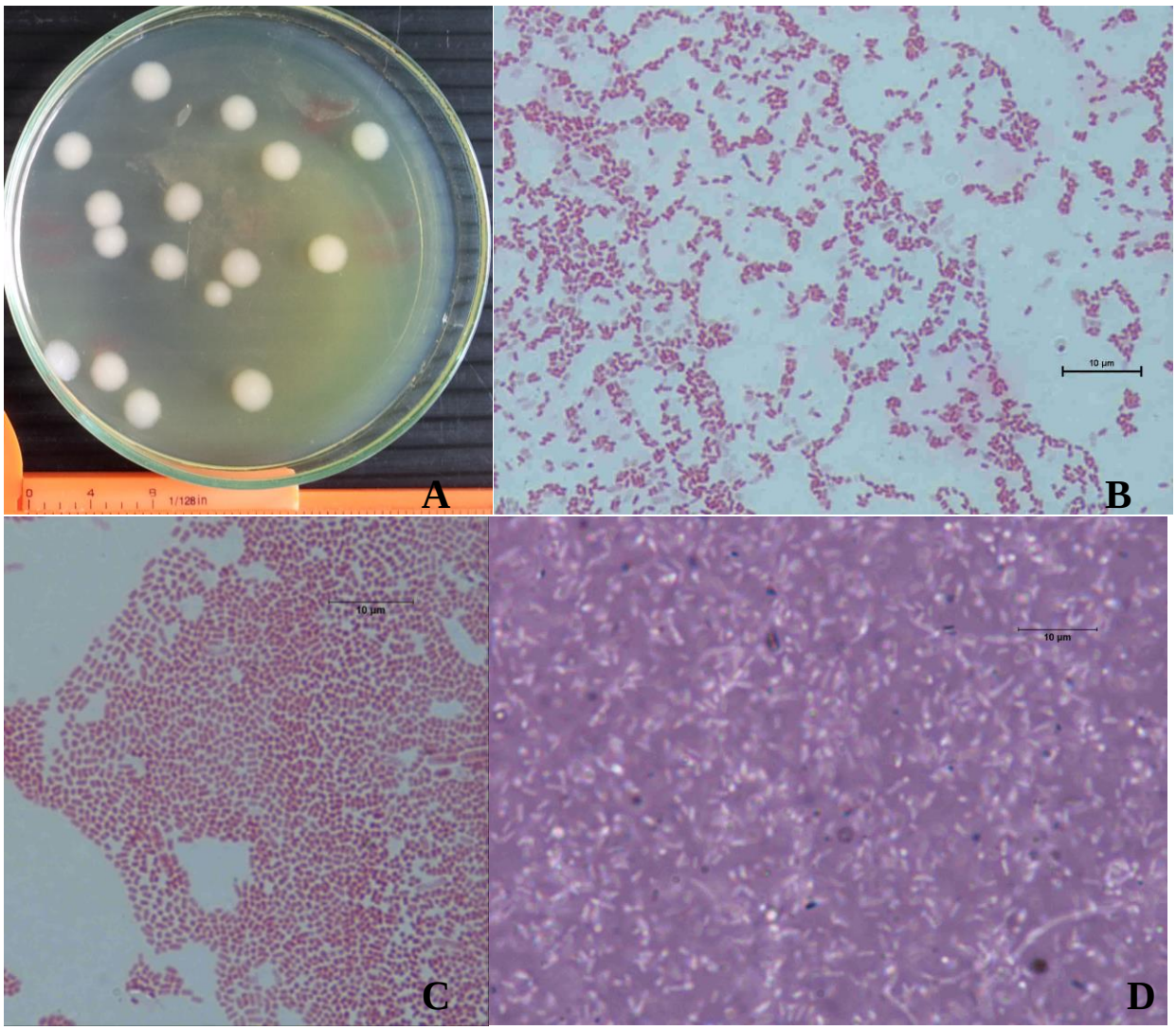

Figure A.4 *Enterobacter* sp. NP02; A: Morphology, B: Gram stain, C: Spore shape and D: Capsule.

Appendix 2: Statistic analysis

Table A.2 Matrix of pairwise comparison probabilities of bacterial isolates adhered to the tilapia epithelial cells at exposure time of 4 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	0.208	1.000	1.000												
B. cereus NP00	1.000	1.000	0.033	1.000											
B. cereus NP01	0.794	1.000	1.000	0.122	1.000										
Bacillus sp. RC00	1.000	1.000	1.000	1.000	1.000	1.000									
Bacillus sp. RC01	1.000	1.000	0.153	1.000	0.584	1.000	1.000								
Bacillus sp. RC02	1.000	0.035	0.001	1.000	0.003	0.231	1.000	1.000							
Bacillus sp. RP00	1.000	1.000	0.299	1.000	1.000	1.000	1.000	0.786	1.000						
Bacillus sp. RP01	1.000	1.000	1.000	0.752	1.000	1.000	1.000	0.014	1.000	1.000					
Enterobactor sp. NP02	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.097	1.000	1.000	1.000				
Enterobacter sp. NP03	1.000	0.518	0.009	1.000	0.032	1.000	1.000	1.000	1.000	0.193	1.000	1.000			
Mac. caseolyticus CHP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.021	1.000	1.000	1.000	0.311	1.000		
Stap. arlettae CHP04	1.000	1.000	0.051	1.000	0.192	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	
Stap. sciuri NP04	0.985	0.030	0.001	1.000	1.000	0.201	1.000	1.000	0.684	0.012	0.085	1.000	0.019	1.000	1.000

Table A.3 Matrix of pairwise comparison probabilities of bacterial isolates adhered to chloroform at exposure time of 30 minutes.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.050	1.000													
Bacillus sp. CHP02	0.000	0.004	1.000												
B. cereus NP00	1.000	0.000	0.000	1.000											
B. cereus NP01	0.537	0.000	0.000	1.000	1.000										
Bacillus sp. RC00	0.222	1.000	0.001	0.001	0.000	1.000									
Bacillus sp. RC01	1.000	0.376	0.000	0.172	0.071	1.000	1.000								
Bacillus sp. RC02	0.000	0.000	0.000	0.006	0.014	0.000	0.000	1.000							
Bacillus sp. RP00	0.318	1.000	0.001	0.001	0.001	1.000	1.000	0.000	1.000						
Bacillus sp. RP01	1.000	1.000	0.000	0.022	0.009	1.000	1.000	0.000	1.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000				
Enterobacter sp. NP03	0.116	0.000	0.000	1.000	1.000	0.000	0.016	0.061	0.000	0.002	0.000	1.000			
Mac. caseolyticus CHP03	1.000	0.636	0.000	0.102	0.043	1.000	0.000	0.000	1.000	1.000	0.000	0.010	1.000		
Stap. arlettae CHP04	0.001	0.000	0.000	0.138	0.336	0.000	1.000	1.000	0.000	0.000	0.000	1.000	0.000	1.000	
Stap. sciuri NP04	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.203	0.000	0.000	0.000	1.000

Table A.4 Matrix of pairwise comparison probabilities of bacterial isolates adhered to hexane at exposure time of 30 minutes.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	1.000	0.024	1.000												
B. cereus NP00	1.000	1.000	0.943	1.000											
B. cereus NP01	1.000	1.000	0.028	1.000	1.000										
Bacillus sp. RC00	1.000	0.410	1.000	1.000	0.471	1.000									
Bacillus sp. RC01	1.000	1.000	1.000	1.000	1.000	1.000	1.000								
Bacillus sp. RC02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000							
Bacillus sp. RP00	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000						
Bacillus sp. RP01	1.000	1.000	0.195	1.000	1.000	1.000	1.000	0.000	1.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000				
Enterobacter sp. NP03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000			
Mac. caseolyticus CHP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.005	1.000	0.000	0.039	1.000	0.002	0.012	0.000	0.015	0.186	0.000	0.000	0.006	1.000	
Stap. sciuri NP04	0.000	0.000	0.010	0.000	0.000	0.001	0.000	0.209	0.000	0.000	0.000	0.000	0.000	0.000	1.000

Table A.5 Matrix of pairwise comparison probabilities of auto-aggregations in PBS of bacterial isolates at exposure time of 4 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	1.000	1.000	1.000												
B. cereus NP00	0.010	0.147	0.003	1.000											
B. cereus NP01	1.000	1.000	1.000	0.019	1.000										
Bacillus sp. RC00	1.000	1.000	1.000	0.015	1.000	1.000									
Bacillus sp. RC01	1.000	1.000	1.000	0.185	1.000	1.000	1.000								
Bacillus sp. RC02	1.000	1.000	1.000	0.001	1.000	1.000	1.000	1.000							
Bacillus sp. RP00	1.000	1.000	1.000	0.600	1.000	1.000	1.000	0.501	1.000						
Bacillus sp. RP01	0.432	1.000	0.095	1.000	0.827	0.674	1.000	0.033	1.000	1.000					
Enterobactor sp. NP02	1.000	1.000	1.000	0.001	1.000	1.000	1.000	1.000	0.465	0.031	1.000				
Enterobacter sp. NP03	1.000	1.000	1.000	0.173	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000			
Mac. caseolyticus CHP03	1.000	1.000	1.000	0.001	1.000	1.000	1.000	1.000	0.749	0.048	1.000	1.000	1.000		
Stap. arlettae CHP04	1.000	1.000	0.959	0.924	1.000	1.000	1.000	0.325	1.000	1.000	0.302	1.000	0.486	1.000	
Stap. sciuri NP04	1.000	0.168	1.000	0.000	1.000	1.000	0.133	1.000	0.042	0.003	1.000	0.143	1.000	0.028	1.000

Table A.6 Matrix of pairwise comparison probabilities of auto-aggregations in PBS of bacterial isolates at exposure time of 6 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	1.000	1.000	1.000												
B. cereus NP00	1.000	0.921	1.000	1.000											
B. cereus NP01	1.000	0.012	0.607	1.000	1.000										
Bacillus sp. RC00	1.000	1.000	1.000	1.000	0.027	1.000									
Bacillus sp. RC01	1.000	1.000	1.000	1.000	1.000	1.000	1.000								
Bacillus sp. RC02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000							
Bacillus sp. RP00	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000						
Bacillus sp. RP01	1.000	0.064	1.000	1.000	1.000	0.143	1.000	0.000	1.000	1.000					
Enterobactor sp. NP02	1.000	0.062	1.000	1.000	1.000	0.139	1.000	0.000	1.000	1.000	1.000				
Enterobacter sp. NP03	0.885	0.010	0.509	1.000	1.000	0.023	1.000	0.000	0.940	1.000	1.000	1.000			
Mac. caseolyticus CHP03	1.000	0.579	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000		
Stap. arlettae CHP04	0.089	1.000	0.154	0.018	0.000	1.000	0.026	0.000	0.084	0.002	0.002	0.000	0.012	1.000	
Stap. sciuri NP04	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.657	0.000	0.000	0.000	0.000	0.000	0.000	1.000

Table A.7 Matrix of pairwise comparison probabilities of auto-aggregations in sterile 0.85% NaCl of bacterial isolates at exposure time of 2 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.205	1.000													
Bacillus sp. CHP02	0.001	1.000	1.000												
B. cereus NP00	0.440	1.000	0.589	1.000											
B. cereus NP01	0.000	0.359	1.000	0.167	1.000										
Bacillus sp. RC00	1.000	1.000	0.085	1.000	0.025	1.000									
Bacillus sp. RC01	0.042	1.000	1.000	1.000	1.000	1.000	1.000								
Bacillus sp. RC02	0.000	0.051	1.000	0.025	1.000	0.004	0.255	1.000							
Bacillus sp. RP00	0.247	1.000	1.000	1.000	0.298	1.000	1.000	0.043	1.000						
Bacillus sp. RP01	1.000	1.000	0.008	1.000	0.003	1.000	0.484	0.000	1.000	1.000					
Enterobactor sp. NP02	0.122	1.000	1.000	1.000	0.601	1.000	1.000	0.085	1.000	1.000	1.000				
Enterobacter sp. NP03	1.000	1.000	0.018	1.000	0.005	1.000	1.000	0.001	1.000	1.000	1.000	1.000			
Mac. caseolyticus CHP03	0.000	0.047	1.000	0.023	1.000	0.004	0.234	1.000	0.039	0.000	0.078	0.001	1.000		
Stap. arlettae CHP04	0.001	0.760	1.000	0.354	1.000	0.052	1.000	1.000	0.631	0.005	1.000	0.011	1.000	1.000	
Stap. sciuri NP04	0.002	1.000	1.000	1.000	1.000	0.249	1.000	1.000	1.000	0.021	1.000	0.049	1.000	1.000	1.000

Table A.8 Matrix of pairwise comparison probabilities of auto-aggregations in sterile 0.85% NaCl of bacterial isolates at exposure time of 4 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	1.000	1.000	1.000												
B. cereus NP00	1.000	1.000	1.000	1.000											
B. cereus NP01	1.000	1.000	1.000	1.000	1.000										
Bacillus sp. RC00	1.000	1.000	1.000	1.000	1.000	1.000									
Bacillus sp. RC01	1.000	1.000	1.000	1.000	1.000	1.000	1.000								
Bacillus sp. RC02	0.011	0.001	0.009	0.001	0.075	0.014	0.001	1.000							
Bacillus sp. RP00	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.166	1.000						
Bacillus sp. RP01	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.006	1.000	1.000					
Enterobactor sp. NP02	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.001	1.000	1.000	1.000				
Enterobacter sp. NP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.041	1.000	1.000	1.000	1.000			
Mac. caseolyticus CHP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.144	1.000	1.000	1.000	1.000	1.000		
Stap. arlettae CHP04	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.053	1.000	1.000	1.000	1.000	1.000	1.000	
Stap. sciuri NP04	0.038	0.004	0.030	0.004	0.278	0.050	0.002	1.000	0.620	0.019	0.003	0.150	0.536	0.195	1.000

Table A.9 Matrix of pairwise comparison probabilities of auto-aggregations in sterile 0.85% NaCl of bacterial isolates at exposure time of 6 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	1.000	1.000	1.000												
B. cereus NP00	1.000	1.000	1.000	1.000											
B. cereus NP01	1.000	1.000	1.000	1.000	1.000										
Bacillus sp. RC00	1.000	1.000	1.000	1.000	1.000	1.000									
Bacillus sp. RC01	1.000	1.000	1.000	1.000	1.000	1.000	1.000								
Bacillus sp. RC02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000							
Bacillus sp. RP00	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000						
Bacillus sp. RP01	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000					
Enterobactor sp. NP02	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000				
Enterobacter sp. NP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000			
Mac. caseolyticus CHP03	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	0.084	1.000	1.000		
Stap. arlettae CHP04	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000	1.000	
Stap. sciuri NP04	0.035	0.014	0.080	0.027	0.149	0.015	0.008	0.004	0.193	0.008	0.002	0.126	0.827	0.294	1.000

Table A.10 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 15^{O}C for 8 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.000	1.000													
Bacillus sp. CHP02	1.000	0.000	1.000												
B. cereus NP00	0.001	0.000	0.010	1.000											
B. cereus NP01	1.000	0.000	1.000	0.000	1.000										
Bacillus sp. RC00	0.908	0.000	1.000	0.227	0.052	1.000									
Bacillus sp. RC01	0.000	0.000	0.000	0.000	0.000	0.000	1.000								
Bacillus sp. RC02	1.000	0.000	0.696	0.000	1.000	0.028	0.000	1.000							
Bacillus sp. RP00	0.172	0.000	1.000	1.000	0.011	1.000	0.000	0.006	1.000						
Bacillus sp. RP01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	1.000				
Enterobacter sp. NP03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000			
Mac. caseolyticus CHP03	0.000	0.000	0.000	1.000	0.000	0.002	0.000	0.000	0.011	0.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.000	0.000	0.001	1.000	0.000	0.017	0.000	0.000	0.087	0.000	0.000	0.000	1.000	1.000	
Stap. sciuri NP04	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	1.000	0.000	0.000	1.000

Table A.11 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 15^{O}C for 24 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	1.000	1.000													
Bacillus sp. CHP02	0.000	0.000	1.000												
B. cereus NP00	0.025	0.348	0.001	1.000											
B. cereus NP01	1.000	1.000	0.000	0.545	1.000										
Bacillus sp. RC00	1.000	1.000	0.000	0.059	1.000	1.000									
Bacillus sp. RC01	0.000	0.000	0.001	0.000	0.000	0.000	1.000								
Bacillus sp. RC02	0.002	0.000	0.000	0.000	0.000	0.001	0.000	1.000							
Bacillus sp. RP00	0.000	0.000	1.000	0.000	0.000	0.000	0.092	0.000	1.000						
Bacillus sp. RP01	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.000	0.223	0.000	0.000	0.223	1.000				
Enterobacter sp. NP03	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	1.000	1.000	1.000			
Mac. caseolyticus CHP03	0.000	0.001	0.223	1.000	0.002	0.000	0.000	0.000	0.002	0.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.000	0.001	0.348	0.850	0.001	0.000	0.000	0.000	0.003	0.000	0.000	0.000	1.000	1.000	
Stap. sciuri NP04	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000	0.000	1.000	1.000	1.000	0.000	0.000	1.000

Table A.12 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 32^{O}C for 8 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.002	1.000													
Bacillus sp. CHP02	1.000	0.035	1.000												
B. cereus NP00	1.000	0.012	1.000	1.000											
B. cereus NP01	1.000	0.008	1.000	1.000	1.000										
Bacillus sp. RC00	0.196	0.000	0.012	0.035	0.053	1.000									
Bacillus sp. RC01	1.000	0.035	1.000	1.000	1.000	0.012	1.000								
Bacillus sp. RC02	0.018	0.000	0.001	0.004	0.005	1.000	0.001	1.000							
Bacillus sp. RP00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000						
Bacillus sp. RP01	0.592	1.000	1.000	1.000	1.000	0.000	1.000	0.000	0.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.028	0.000	0.000	1.000				
Enterobacter sp. NP03	1.000	0.000	1.000	1.000	1.000	1.000	1.000	0.305	0.000	0.035	0.000	1.000			
Mac. caseolyticus CHP03	1.000	0.305	1.000	1.000	1.000	0.002	1.000	0.000	0.000	1.000	0.000	0.158	1.000		
Stap. arlettae CHP04	1.000	0.000	0.381	1.000	1.000	1.000	0.381	1.000	0.000	0.010	0.000	1.000	0.043	1.000	
Stap. sciuri NP04	1.000	0.000	0.102	0.305	0.475	1.000	0.102	1.000	0.000	0.003	0.000	1.000	0.012	1.000	1.000

Table A.13 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 32^{O}C for 24 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.002	1.000													
Bacillus sp. CHP02	0.002	1.000	1.000												
B. cereus NP00	0.007	1.000	1.000	1.000											
B. cereus NP01	1.000	0.003	0.003	0.014	1.000										
Bacillus sp. RC00	0.000	0.002	0.002	0.000	0.000	1.000									
Bacillus sp. RC01	0.723	0.723	0.723	1.000	1.000	0.000	1.000								
Bacillus sp. RC02	1.000	0.000	0.000	0.001	1.000	0.000	0.066	1.000							
Bacillus sp. RP00	0.000	0.000	0.000	0.000	0.000	0.723	0.000	0.000	1.000						
Bacillus sp. RP01	0.030	1.000	1.000	1.000	0.066	0.000	1.000	0.003	0.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000				
Enterobacter sp. NP03	0.723	0.000	0.000	0.000	0.324	0.000	0.002	1.000	0.000	0.000	0.003	1.000			
Mac. caseolyticus CHP03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.066	0.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.000	0.000	0.000	0.000	0.000	0.146	0.000	0.000	1.000	0.000	0.000	0.000	1.000	1.000	
Stap. sciuri NP04	0.324	0.000	0.000	0.000	0.146	0.000	0.001	1.000	0.000	0.000	0.007	1.000	0.000	0.000	1.000

Table A.14 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 42^{O}C for 8 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.000	1.000													
Bacillus sp. CHP02	0.000	0.981	1.000												
B. cereus NP00	0.000	0.012	1.000	1.000											
B. cereus NP01	0.193	0.000	0.001	0.086	1.000										
Bacillus sp. RC00	0.000	0.000	0.000	0.000	0.000	1.000									
Bacillus sp. RC01	0.058	0.000	0.004	0.289	1.000	0.000	1.000								
Bacillus sp. RC02	0.004	0.000	0.058	1.000	1.000	0.000	1.000	1.000							
Bacillus sp. RP00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000						
Bacillus sp. RP01	0.058	0.000	0.004	0.289	1.000	0.000	1.000	1.000	0.000	1.000					
Enterobactor sp. NP02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000				
Enterobacter sp. NP03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.058	1.000			
Mac. caseolyticus CHP03	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.000	0.000	0.000	0.000	0.000	0.128	0.000	0.000	0.193	0.000	0.000	0.000	0.006	1.000	
Stap. sciuri NP04	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.193	1.000	0.000	0.000	1.000

Table A.15 Matrix of pairwise comparison probabilities of specific growth rates of bacterial isolates at exposure temperature of 42^{O}C for 24 hours.

	B. cereus CHP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Bacillus* sp. RP00	*Bacillus* sp. RP01	*Enterobactor* sp. NP02	*Enterobacter* sp. NP03	*Mac. caseolyticus* CHP03	*Stap. arlettae* CHP04	*Stap. sciuri* NP04
B. cereus CHP00	1.000														
Bacillus sp. CHP01	0.046	1.000													
Bacillus sp. CHP02	1.000	1.000	1.000												
B. cereus NP00	0.001	1.000	0.046	1.000											
B. cereus NP01	0.002	1.000	0.084	1.000	1.000										
Bacillus sp. RC00	0.000	0.000	0.000	0.003	0.002	1.000									
Bacillus sp. RC01	0.002	1.000	0.084	1.000	1.000	0.002	1.000								
Bacillus sp. RC02	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.000							
Bacillus sp. RP00	0.000	0.000	0.000	0.000	0.000	0.284	0.000	0.000	1.000						
Bacillus sp. RP01	0.524	1.000	1.000	0.524	0.965	0.000	0.965	0.000	0.000	1.000					
Enterobactor sp. NP02	1.000	0.000	0.025	0.000	0.000	0.000	0.000	0.000	0.000	0.003	1.000				
Enterobacter sp. NP03	0.284	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.001	1.000	1.000			
Mac. caseolyticus CHP03	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.524	0.000	0.000	0.000	1.000		
Stap. arlettae CHP04	0.000	0.000	0.000	0.000	0.000	0.046	0.000	0.000	1.000	0.000	0.000	0.000	1.000	1.000	
Stap. sciuri NP04	0.284	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000	0.001	1.000	1.000	0.000	0.000	1.000

Appendix 3: The method of Z-score calculations

Step 1: Samples are calculated to get scores of parameter studies by using conditions of I to VI (Chapter 3), which display in **Table A.17**.

Step 2: Samples are calculated T_i score by using parameter properties to multiply with the coefficient index in Table 3.1. Scores and overall mean ($\bar{T}$) are represented in **Table A.18**.

Step 3: The Z-score equation is broken down to find $(T_i - \bar{T})$, and $(T_i - \bar{T})^2$ and then these items are calculated (**Table A.19 & A.20**).

$$Z_i = \frac{\sum_i (T_i - \bar{T})}{\sqrt{\frac{\sum_1^n (T_i - \bar{T})^2}{n-1}}}$$

Step 4: In **Table A.20**, calculate as $\sqrt{\frac{\sum_1^n (T_i - \bar{T})^2}{n-1}} = \sqrt{\frac{12563.39}{14}} = 29.956$

Step 4: Finally, isolates are estimated Z-scores in **Table A.21**.

Where: T_i is the total score of isolated bacterial i, $\bar{T}$ is the overall mean score, and n is the total isolate number.

Table A.16 Represent scores of antibiotic resistance of isolates.

Antibiotic disc	**Bacterial isolates**														
	Bacillus sp. RP01	*B. cereus* CHP00	*B. cereus* NP00	*B. cereus* NP01	*Bacillus* sp. RP00	*Bacillus* sp. CHP01	*Bacillus* sp. CHP02	*Bacillus* sp. RC00	*Bacillus* sp. RC01	*Bacillus* sp. RC02	*Enterobacter* sp. NP03	*Enterobactor* sp. NP02	*Mac. caseolyticus* CHP03	*Stap.arlettae* CHP04	*Stap. sciuri* NP04
Total count of S	12	11	11	11	12	11	12	12	12	11	10	11	11	12	12
Total count of I	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Total count of R	0	1	1	1	0	3	0	0	0	1	1	1	1	0	0
Scores of S	100	91.7	91.7	91.7	100	91.7	100	100	100	91.7	83.3	91.7	91.7	100	100
Scores of I	0				0	0	0	-50	0		-50		0	0	0
Scores of R	0	−33.3	−33.3	−33.3	0	−100	0	0	0	−33.3	−33.3	−33.3	−33.3	0	0
Total scores	**100**	**58**	**58**	**58**	**100**	**-8**	**100**	**50**	**100**	**58**	**0**	**58**	**58**	**100**	**100**

Table A.17 Represent scores of isolates by using results of *in vitro* trials.

Bacterial isolates	**Pathogenic inhibition**	**Adhesion to the tilapia epithelial cells**	**Adhesion to hydrocarbon solvents**	**Auto-aggregation**	**Antibiotic susceptibility test**	**Hemolysis**	**Bile salt tolerance**	**Acid tolerance**	**Temperature exposures**
Bacillus sp. RP01	50.0	82.0	47.2	29.0	100.0	100	100	100	96.43
B. cereus CHP00	100.0	61.4	52.9	27.3	58.3	-100	50	100	66.42
B. cereus NP00	100.0	37.4	44.8	20.7	58.3	-100	50	100	75.63
B. cereus NP01	100.0	86.5	65.6	18.0	58.3	-100	50	100	68.14
Bacillus sp. RP00	50.0	62.7	56.8	33.2	100.0	100	50	100	90.57
Bacillus sp. CHP01	100.0	62.6	51.7	32.4	-8.3	-100	50	100	100.00
Bacillus sp. CHP02	100.0	100.0	62.3	24.3	100.0	100	50	100	74.01
Bacillus sp. RC00	100.0	62.3	52.8	34.8	50.0	100	100	100	75.81
Bacillus sp. RC01	50.0	45.3	52.3	28.6	100.0	100	50	100	86.68
Bacillus sp. RC02	50.0	20.7	92.8	23.4	58.3	-100	50	100	67.24
Enterobacter sp. NP03	50.0	33.3	56.2	2.3	0.0	100	100	0	89.24
Enterobactor sp. NP02	100.0	66.2	55.7	100.0	58.3	100	100	0	76.76
Mac. caseolyticus CHP03	50.0	71.9	68.7	29.5	58.3	100	50	0	88.42
Stap. arlettae CHP04	50.0	21.3	78.7	8.4	100.0	100	100	0	83.54
Stap. sciuri NP04	50.0	41.0	67.8	57.5	100.0	100	100	0	88.60

Table A.18 Represent scores of isolates after using scores (Table A.17) multiply with coefficient index.

Bacterial isolates	**Pathogenic inhibition**	**Adhesion to the tilapia epithelial cells**	**Adhesion to hydrocarbon solvents**	**Auto-aggregation**	**Antibiotic susceptibility test**	**Hemolysis**	**Bile salt tolerance**	**Acid tolerance**	**Temperature exposures**	
Bacillus sp. RP01	0.03	0.15	0.06	0.06	0.25	0.25	0.04	0.10	0.06	
B. cereus CHP00	1.50	12.31	2.83	1.74	25.00	25.00	4.00	10.00	5.79	
B. cereus NP00	3.00	9.22	3.17	1.64	14.58	-25.00	2.00	10.00	3.98	
B. cereus NP01	3.00	5.61	2.69	1.24	14.58	-25.00	2.00	10.00	4.54	
Bacillus sp. RP00	3.00	12.98	3.94	1.08	14.58	-25.00	2.00	10.00	4.09	
Bacillus sp. CHP01	1.50	9.40	3.41	1.99	25.00	25.00	2.00	10.00	5.43	
Bacillus sp. CHP02	3.00	9.39	3.10	1.95	-2.08	-25.00	2.00	10.00	6.00	
Bacillus sp. RC00	3.00	15.00	3.74	1.46	25.00	25.00	2.00	10.00	4.44	
Bacillus sp. RC01	3.00	9.34	3.17	2.09	12.50	25.00	4.00	10.00	4.55	
Bacillus sp. RC02	1.50	6.79	3.14	1.71	25.00	25.00	2.00	10.00	5.20	
Enterobacter sp. NP03	1.50	3.11	5.57	1.41	14.58	-25.00	2.00	10.00	4.03	
Enterobactor sp. NP02	1.50	4.99	3.37	0.14	0.00	25.00	4.00	0.00	5.35	
Mac. caseolyticus CHP03	3.00	9.93	3.34	6.00	14.58	25.00	4.00	0.00	4.61	
Stap. arlettae CHP04	1.50	10.78	4.12	1.77	14.58	25.00	2.00	0.00	5.30	
Stap. sciuri NP04	1.50	3.19	4.72	0.50	25.00	25.00	4.00	0.00	5.01	
Mean	2.20	8.55	3.63	1.88	16.53	8.33	2.80	6.67	4.91	**6.17** Onerall mean

Table A.19 Representation of $'T_i - \overline{T}'$ calculation by using scores in Table A.18 minus with overall mean.

Bacterial isolates	**Pathogenic inhibition**	**Adhesion to the tilapia epithelial cells**	**Adhesion to hydrocarbon solvents**	**Auto-aggregation**	**Antibiotic susceptibility test**	**Hemolysis**	**Bile salt tolerance**	**Acid tolerance**	**Temperature exposures**	**Summation**
Bacillus sp. RP01	−4.67	6.14	−3.34	−4.42	18.84	18.84	−2.17	3.84	−0.38	32.68
B. cereus CHP00	−3.17	3.05	−2.99	−4.53	8.42	−31.17	−4.17	3.84	−2.18	−32.89
B. cereus NP00	−3.17	−0.56	−3.48	−4.92	8.42	−31.17	−4.17	3.84	−1.63	−36.82
B. cereus NP01	−3.17	6.82	−2.23	−5.08	8.42	−31.17	−4.17	3.84	−2.08	−28.82
Bacillus sp. RP00	−4.67	3.23	−2.76	−4.17	18.84	18.84	−4.17	3.84	−0.73	28.25
Bacillus sp. CHP01	−3.17	3.22	−3.06	−4.22	−8.25	−31.17	−4.17	3.84	−0.17	−47.13
Bacillus sp. CHP02	−3.17	8.84	−2.43	−4.71	18.84	18.84	−4.17	3.84	−1.72	34.15
Bacillus sp. RC00	−3.17	3.17	−3.00	−4.08	6.34	18.84	−2.17	3.84	−1.62	18.16
Bacillus sp. RC01	−4.67	0.63	−3.03	−4.45	18.84	18.84	−4.17	3.84	−0.96	24.86
Bacillus sp. RC02	−4.67	−3.05	−0.60	−4.76	8.42	−31.17	−4.17	3.84	−2.13	−38.28
Enterobacter sp. NP03	−4.67	−1.17	−2.79	−6.03	−6.17	18.84	−2.17	−6.17	−0.81	−11.13
Enterobactor sp. NP02	−3.17	3.77	−2.82	−0.17	8.42	18.84	−2.17	−6.17	−1.56	14.98
Mac. caseolyticus CHP03	−4.67	4.61	−2.04	−4.39	8.42	18.84	−4.17	−6.17	−0.86	9.57
Stap. arlettae CHP04	−4.67	−2.97	−1.44	−5.66	18.84	18.84	−2.17	−6.17	−1.15	13.45
Stap. sciuri NP04	−4.67	−0.02	−2.10	−2.72	18.84	18.84	−2.17	−6.17	−0.85	18.99

Table A.20 Representation calculate to square of $'(T_i - \bar{T})^{2'}$ by using scores in Table A.19.

Bacterial isolates	**Pathogenic inhibition**	**Adhesion to the tilapia epithelial cells**	**Adhesion to hydrocarbon solvents**	**Auto-aggregation**	**Antibiotic susceptibility test**	**Hemolysis**	**Bile salt tolerance**	**Acid tolerance**	**Temperature exposures**	**Summation**
Bacillus sp. RP01	21.76	37.71	11.13	19.56	354.76	354.76	4.69	14.71	0.14	819.21
B. cereus CHP00	10.02	9.31	8.96	20.49	70.87	971.26	17.35	14.71	4.75	1127.71
B. cereus NP00	10.02	0.31	12.09	24.21	70.87	971.26	17.35	14.71	2.65	1123.46
B. cereus NP01	10.02	46.45	4.97	25.85	70.87	971.26	17.35	14.71	4.31	1165.77
Bacillus sp. RP00	21.76	10.45	7.59	17.39	354.76	354.76	17.35	14.71	0.53	799.30
Bacillus sp. CHP01	10.02	10.37	9.37	17.80	68.04	971.26	17.35	14.71	0.03	1118.93
Bacillus sp. CHP02	10.02	78.06	5.89	22.15	354.76	354.76	17.35	14.71	2.97	860.65
Bacillus sp. RC00	10.02	10.07	8.99	16.63	40.13	354.76	4.69	14.71	2.61	462.60
Bacillus sp. RC01	21.76	0.40	9.16	19.81	354.76	354.76	17.35	14.71	0.93	793.62
Bacillus sp. RC02	21.76	9.33	0.36	22.64	70.87	971.26	17.35	14.71	4.54	1132.80
Enterobacter sp. NP03	21.76	1.37	7.79	36.34	38.01	354.76	4.69	38.01	0.66	503.38
Enterobactor sp. NP02	10.02	14.18	7.96	0.03	70.87	354.76	4.69	38.01	2.43	502.94
Mac. caseolyticus CHP03	21.76	21.28	4.18	19.31	70.87	354.76	17.35	38.01	0.74	548.26
Stap. arlettae CHP04	21.76	8.83	2.08	32.08	354.76	354.76	4.69	38.01	1.33	818.28
Stap. sciuri NP04	21.76	0.00	4.40	7.38	354.76	354.76	4.69	38.01	0.72	786.48
										12563.39

Table A.21 Represent of Z-score calculation of isolates.

Bacterial isolates	$\mathbf{Z-scores} = \frac{\sum_i (T_i - \bar{T}) *}{\sqrt{\frac{\sum_1^n (T_i - \bar{T})^{2**}}{n-1}}}$
Bacillus sp. RP01	(32.68⁄29.96) = 1.09
B. cereus CHP00	(-32.89⁄29.96) = –1.10
B. cereus NP00	(-36.82⁄29.96) = –1.23
B. cereus NP01	(-28.82⁄29.96) = –0.96
Bacillus sp. RP00	(28.25⁄29.96) = 0.94
Bacillus sp. CHP01	(-47.13⁄29.96) = –1.57
Bacillus sp. CHP02	(34.15⁄29.96) = 1.14
Bacillus sp. RC00	(18.16⁄29.96) = 0.61
Bacillus sp. RC01	(24.86⁄29.96) = 0.83
Bacillus sp. RC02	(-38.28⁄29.96) = –1.28
Enterobacter sp. NP03	(-11.13⁄29.96) = –0.37
Enterobactor sp. NP02	(14.98⁄29.96) = 0.50
Mac. caseolyticus CHP03	(9.57⁄29.96) = 0.32
Stap. arlettae CHP04	(13.45⁄29.96) = 0.45
Stap. sciuri NP04	(18.99⁄29.96) = 0.63

* in Table A.19

** in Table A.20

$\sqrt{\frac{\sum_1^n (T_i - \bar{T})^2}{n-1}} = \sqrt{\frac{12563.39}{14}} = 29.956$

Appendix 4: Training and courses attended to date

- Originality and plagiarism (Wednesday 11st December 2013)
- Scientific Writing Skills Course (Friday 7th March 2014)
- The Transfer Process (Friday 14th March 2014)
- Academic writing workshop (Wednesday 12nd February 2014)
- Introduction to R (Wednesday 8th January 2014)
- Presenting at conferences (Thursday 5th February 2015)
- Preparing for VIVA (Tuesday 8th March 2016)
- Preparing to submit on Pearl including copyright and open access (Wednesday 10th March 2016)